Cyprus University of Technology

Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

The effect of pre- and postharvest factors on qualitative attributes, phytochemical properties and incidence of physiological disorders in loquat fruit

Margarita Hadjipieri, MSc

Limassol, December 2020

CYPRUS UNIVERSITY OF TECHNOLOGY
FACULTY OF GEOTECHNICAL SCIENCES AND ENVIROMENTAL MANAGEMENT
DEPARTMENT OF AGRICULTURAL SCIENCES, BIOTECHNOLOGY AND FOOD SCIENCE

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Approval Form

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Declaration

The data presented in this PhD dissertation are the results of an original research work conducted by the author at the Cyprus University of Technology (CUT), unless otherwise indicated. The work contained herein has not been submitted, in whole or in part, to obtain any other degree or professional qualification in this or any other academic institution. Data of this study have been published or submitted in four peer-reviewed journals. In addition, part of the data of the current dissertation have been defended as oral presentations by Mrs Hadjipieri in two conferences, held under the auspices of the Hellenic Society for Horticultural Science and the International Society for Horticultural Science, respectively.

Scientific publications in referred journals

1. Hadjipieri M., Georgiadou E.C, Marin A., Diaz-Mula H.M, Goulas V, Fotopoulos V, Tomás-Barberán F.A, Manganaris G.A. (2017) 'Metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway reveals distinct regulation patterns between peel and flesh tissue of loquat fruit during on-tree development'. BMC Plant Biology 17:102.
2. Hadjipieri M., Christofi M., Goulas V., Manganaris G.A., (2020) 'The impact of harvesting day on mechanical properties, qualitative attributes and postharvest performance of two loquat cultivars'. Scientia Horticulturae, 261:108891.
3. Hadjipieri M., Georgiadou E.C, Costa F., Fotopoulos V, Manganaris G.A. (2020) 'Dissection of the incidence and severity of purple spot physiological disorder in loquat fruit through a physiological and molecular approach'. Plant Physiology Biochemistry, 155, 980-986.
4. Hadjipieri M., Georgiadou E.C, Dogoudi P., Fotopoulos V, Manganaris G.A. (2020) 'The efficacy of preharvest foliar spray applications on yield efficiency, incidence of physiological disorders and shelf life performance of loquat fruit'. Manuscript in preparation.

Conference proceedings

1. Hadjipieri M., Gavriel K., G. Sismanidis G., G.A. Manganaris G.A. (2019) 'The effect of modified atmosphere packaging on postharvest performance of two loquat cultivars'. Acta Hortculturae.1242: 729-733.

Oral presentations

1. 'The effect of modified atmosphere packaging on the postharvest performance of two loquat cultivars in cold storage'. 27th Congress of the Greek Society of Horticulture (page 93), 28th September-1st October 2015.
2. 'Metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway reveals distinct regulation patterns between exocarp and mesocarp tissue of loquat fruit during on-tree development'. III International Symposium on Horticulture in Europe (page 76), 17-22 October 2016.

Poster presentations

1. 'The effect of modified atmosphere packaging on postharvest performance of two loquat cultivars' III International Symposium on Horticulture in Europe (page 201), 17-22 October 2016.
2. 'Carotenoid metabolism of loquat fruit (*Eriobotrya japonica*, cv. 'Obusa') during on-tree development. 27th Congress of the Greek Society of Horticulture (page 131), 28th September-1st October 2015.

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GREEK ABSTRACT

Η μεσπιλιά (*Eriobotrya japonica* Lindl.) ανήκει στην οικογένεια Rosaceae με καταγωγή από την Κίνα. Είναι ένα υποτροπικό αειθαλές οπωροφόρο δένδρο που ανθίζει από το φθινόπωρο έως τις αρχές του χειμώνα και παράγει καρπούς στις αρχές της άνοιξης, ανάλογα με την ποικιλία και το σύστημα καλλιέργειας. Ο αντίστροφος ετήσιος κύκλος του, επιτρέπει στο είδος να προσαρμόζεται ικανοποιητικά στη λεκάνη της Μεσογείου, καθώς και σε άλλα υποτροπικά κλίματα αντίστοιχα με αυτά που ευνοούν την εσπεριδοκαλλιέργεια. Τα τελευταία χρόνια η παγκόσμια καλλιέργεια μεσπιλιάς έχει εντατικοποιηθεί λόγω των εξαιρετικών οργανοληπτικών χαρακτηριστικών του καρπού, της υψηλής θρεπτικής αξίας καθώς και των μειωμένων φυτοϋγειονομικών προβλημάτων. Στην Κύπρο, η καλλιέργεια της μεσπιλιάς ευνοείται κυρίως στις παράκτιες περιοχές, με κάλυψη από δίκτυ ή/και πλαστικό. Η παραγωγή βασίζεται κατά κύριο λόγο στις ποικιλίες 'Καραντώκη' και 'Μορφίτικη' και προορίζεται αποκλειστικά για εγχώρια κατανάλωση λόγω του χαμηλού όγκου παραγωγής και της βραχείας μετασυλλεκτικής διάρκειας ζωής.

Η εμπορική παραγωγή της μεσπιλιάς προκαλεί συνεχώς αυξανόμενο ενδιαφέρον καθώς αποτελεί ένα εξειδικευμένο προϊόν που σήμερα μπορεί να κερδίσει προστιθέμενη αξία, αφού διατίθεται κατά τη διάρκεια της περιόδου του χειμώνα-αρχές της άνοιξης όταν δεν υπάρχουν άλλοι καρποί προς διάθεση αυτή την περίοδο. Υπό το πρίσμα αυτό, η παρούσα διατριβή αποτελείται από πέντε ανεξάρτητα και παράλληλα αλληλένδετα πειράματα. Οι δύο πρώτες μελέτες αφορούσαν επίπεδο βασικής έρευνας και περιελάμβαναν: (1) την εξέταση του προφίλ των καροτενοειδών στην επιδερμίδα και την σάρκα σε διαδοχικά αναπτυξιακά στάδια του καρπού και (2) τη διερεύνηση του ρόλου των γονιδίων που σχετίζονται με το βιοσυνθετικό μονοπάτι των πολυφαινολών (*PAL1*, *PAL2* και *PPO1*) και των υδατανθράκων (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* και *VI*) στην ανάπτυξη της φυσιολογικής διαταραχής του ιώδους μεταχρωματισμού. Επιπλέον, πραγματοποιήθηκαν τρία πειράματα εφαρμοσμένης έρευνας: (1) η επίδραση της ημερομηνίας συγκομιδής στα φυτοχημικά χαρακτηριστικά, τις μηχανικές ιδιότητες και τη μετασυλλεκτική συμπεριφορά των δύο κυριότερων ποικιλιών μεσπιλιάς που καλλιεργούνται στην Κύπρο, (2) τη επίδραση διαφυλλικών ψεκασμών με ακετυλοσαλικυλικό οξύ, σπερμιδίνη, και ενός εμπορικού σκευάσματος ασβεστίου στη μετασυλλεκτική συμπεριφορά του καρπού και (3) την επίδραση της

τροποποιημένης ατμόσφαιρας κατά τη συσκευασία στη μετασυλλεκτική συμπεριφορά καρπών δυο ποικιλιών μεσπιλιάς μετά από ψυχρή συντήρησή τους.

Αρχικά, μελετήθηκε η μεταβολική και μεταγραφική μεταβολή της έκφρασης του βιοσυνθετικού μονοπατιού των καροτενοειδών. Τα αποτελέσματα έδειξαν διακριτά πρότυπα ρύθμισης μεταξύ ιστού επιδερμίδας και σάρκας της ποικιλίας 'Obusa' κατά την διάρκεια διαδοχικών σταδίων ανάπτυξης του καρπού. Στην παρούσα εργασία επιπρόσθετα επιχειρήθηκε συσχέτιση μεταξύ του βαθμού ρύθμισης (επαγωγής/καταστολής) της γονιδιακής έκφρασης στα διαδοχικά αναπτυξιακά στάδια του καρπού μεσπιλιάς και της περιεκτικότητάς σε καροτενοειδή του κάθε ιστού. Γενικά παρατηρήθηκαν ουσιαστικές μεταβολές όσον αφορά τους χρωματικούς δείκτες τόσο στην επιδερμίδα όσο και στην σάρκα, καθώς επίσης και στα διαδοχικά στάδια ανάπτυξης, ταυτόχρονα με σημαντική αύξηση της περιεκτικότητας σε καροτενοειδή με την εξέλιξη της ανάπτυξης του καρπού. Τα γονίδια (*DXS, DXR, PSY1, PDS, ZDS, CRTISO, LCYB, CYCB, LCYE, BCH, ECH, ZEP* και *VDE*) και καροτενοειδή τα οποία εμπλέκονται στο βιοσυνθετικό μονοπάτι των καροτενοειδών αναλύθηκαν περαιτέρω. Τριάντα δύο καροτενοειδή βρέθηκαν στη επιδερμίδα, ενώ δεκαοκτώ εντοπίστηκαν στη σάρκα. Τα κύρια καροτενοειδή στη επιδερμίδα ήταν η *trans*-λουτεΐνη και το *trans*-β-καροτένιο. Το περιεχόμενο των πρώτων μειώθηκε με την πρόοδο της ανάπτυξης του καρπού ενώ τα τελευταία κατέγραψαν 7,2 φορές αύξηση. Το προφίλ των καροτενοειδών στην σάρκα υπέδειξε την *trans*-β-κρυπτοξανθίνη, ακολουθούμενη από το *trans*-β-καροτένιο και 8-*epoxy*-β-καροτένιο να κυριαρχούν. Τα αποτελέσματα των υψηλών συγκεντρώσεων *trans*-λουτεΐνη και *trans*-β-καροτένιο υποστηρίζονται από την σημαντική επαγωγή των γονιδίων *BCH* και *CYCB,* ενώ οι μειωμένες συγκεντρώσεις *trans*-λουτεΐνης στη σάρκα σε σχέση με την επιδερμίδα συσχετίζονται με σημαντική καταστολή των γονιδίων *LCYB* και *LCYE*.

Η φυσιολογική διαταραχή του ιώδους μεταχρωματισμού (με ιδιαίτερα εμφανή συμπτώματα στο στάδιο της εμπορικής ωριμότητας) επηρεάζει σημαντικά την εμπορευσιμότητα καρπών μεσπιλιάς. Στην παρούσα μελέτη διερευνήθηκε ο ρόλος των σακχάρων της επιδερμίδας, καθώς και των γονιδίων που εμπλέκονται στο βιοσυνθετικό μονοπάτι του χλωρογενικού οξέος και της σουκρόζης στην ανάπτυξη της διαταραχής. Οι καρποί που συλλέχθηκαν χωρίστηκαν σε πέντε διακριτές ομάδες με βάση την ένταση των συμπτωμάτων. Οι καρποί της ποικιλίας 'Obusa' εμφάνισαν την υψηλότερη

εμφάνιση συμπτωμάτων (58,6% επί του συνόλου των συγκομισμένων καρπών), ακολουθούμενη από την ποικιλία ‘Καραντώκη’ (31,3%), ενώ δεν καταγράφηκαν συμπτώματα στην ποικιλία ‘Μορφιτική’.

Αυξημένα επίπεδα συνολικών διαλυτών σακχάρων, σουκρόζης, γλυκόζης και φρουκτόζης καταγράφηκαν στον ιστό επιδερμίδας των καρπών της ποικιλίας ‘Καραντώκη’ με συμπτώματα ιώδους μεταχρωματισμού. Η χαμηλή περιεκτικότητα σε συνολικά διαλυτά σάκχαρα, γλυκόζη και φρουκτόζη καταγράφηκε σε ασυμπτωματικό ιστό επιδερμίδας της ποικιλίας ‘Obusa’. Στη συνέχεια μελετήθηκε ο ρόλος των γονιδίων του μονοπατιού του χλωρογενικού οξέος (*PAL1, PAL2, C3H* και *PPO1*) και της βιοσύνθεσης της σουκρόζης (*CWI2, CWI3, SPS, SPS1, SPS2, NI2, NI3, SUSY, HXK, FRK* και *VI*) στην ένταση της εμφάνισης του ιώδους μεταχρωματισμού στις υπό εξέταση ποικιλίες. Τα αποτελέσματα έδειξαν ότι η γονιδιακή έκφραση των *CWI3* και *VI* προκύπτει να συσχετίζεται με το φαινόμενο του ιώδους μεταχρωματισμού στους καρπούς της ποικιλίας ‘Obusa’ και προτείνετε περαιτέρω αξιολόγηση τους για διερεύνηση της πιθανής χρήσης τους ως δείκτες σε προγράμματα γενετικής βελτίωσης μεσπιλιάς.

Η μεσπιλιά χαρακτηρίζεται από διαδοχική ωρίμανση των καρπών. Για το λόγο αυτό μελετήθηκε η επίδραση της ημερομηνίας συγκομιδής στις μηχανικές ιδιότητες, τη μετασυλλεκτική συμπεριφορά και τα φυτοχημικά χαρακτηριστικά (ελεύθερες και δεσμευμένες φαινολικές ενώσεις και αντιοξειδωτική ικανότητα) των δύο κυριότερων ποικιλιών μεσπιλιάς (‘Μορφίτικη’, ‘Καραντώκη’) στην Κύπρο. Τα παραπάνω χαρακτηριστικά προσδιορίστηκαν κατά τη συγκομιδή και μετά από επιπλέον διατήρηση σε θερμοκρασία δωματίου για 3 ημέρες για τέσσερις διαδοχικές ημερομηνίες συγκομιδής. Η συνεκτικότητα της σάρκας ήταν ελαφρώς υψηλότερη κατά την πρώτη συγκομιδή σε σύγκριση με τις υπόλοιπες, ενώ μικρές ή καθόλου διαφορές παρατηρήθηκαν κατά την διατήρηση των καρπών στο ράφι και για τις δύο ποικιλίες. Οι καρποί της ποικιλίας ‘Καραντώκη’ παρουσίασαν αυξημένες τιμές δείκτη ωρίμανσης σε σχέση με τους καρπούς της ποικιλίας ‘Μορφίτικης’. Αυτές οι τιμές ήταν υψηλότερες με την πάροδο της ημερομηνίας συγκομιδής λόγω σημαντικής μείωσης της τιτλοδοτούμενης οξύτητας. Δεν καταγράφηκαν εμφανείς διαφορές μεταξύ της ημερομηνίας συγκομιδής και της διατήρησης στο ράφι σε σχέση με την περιεκτικότητα ελεύθερων φαινολικών συστατικών. Τα αποτελέσματα δίνουν έμφαση στην σημασία των δεσμευμένων φαινολικών που συνέβαλαν στο ολικό φαινολικό περιεχόμενο των

καρπών μεσπιλιάς κατά 21,6-37,5%, ανάλογα με την ημερομηνία συγκομιδής, της ποικιλίας και της μετασυλλεκτικής μεταχείρισης που εφαρμόστηκε. Αυτή η μελέτη παρέχει νέα πληροφόρηση σχετικά με το ρόλο των δεσμευμένων φυτοχημικών ιδιοτήτων των καρπών μεσπιλιάς.

Η επίδραση διαφυλλικών ψεκασμών με ακετυλοσαλικυλικό οξύ, σπερμιδίνη, και ενός εμπορικού προϊόντος ασβεστίου αξιολογήθηκε ως προς την επίδραση τους στην απόδοση παραγωγής, τα ποιοτικά χαρακτηριστικά και τη μετασυλλεκτική ζωή στο ράφι καθώς και στην εμφάνιση φυσιολογικών διαταραχών (ιώδους μεταχρωματισμού και σχισίματος καρπού από έντονη βροχόπτωση) σε καρπούς μεσπιλιάς. Οι διαφυλλικοί ψεκασμοί δεν επηρέασαν την ανάπτυξη των καρπών και την συνολική απόδοση των δένδρων. Το ακετυλοσαλικυλικό οξύ προτείνεται ως μία οικονομικά συμφέρουσα χημική ουσία η οποία θα πρέπει να διερευνηθεί περαιτέρω για την χρησιμότητά της ως προς την παραγωγή ποιοτικών καρπών. Αντίθετα, η διαφυλλική εφαρμογή της σπερμιδίνης μόνη της ή σε συνδυασμό με το ακετυλοσαλικυλικό οξύ δεν έδειξε ουσιαστική βελτίωση στα ποιοτικά χαρακτηριστικά των καρπών ώστε να δικαιολογεί το κόστος εφαρμογής της. Με βάση τα αποτελέσματα της οικονομικής ανάλυσης η μελέτη υποδεικνύει ότι η χρήση του ακετυλοσαλικυλικού οξέος μόνο του ή σε συνδυασμό με εμπορικό προϊόν ασβεστίου, βελτιώνει τα ποιοτικά χαρακτηριστικά των καρπών και προσδίδει προστιθέμενη αξία στο προϊόν. Για το σκοπό αυτό, απαιτείται περαιτέρω διερεύνηση των αναφερόμενων διαφυλλικών ψεκασμών κάτω από διαφορετικές περιβαλλοντικές συνθήκες, οι οποίες μπορεί να επιταχύνουν την εμφάνιση των φυσιολογικών διαταραχών ή/και εφαρμογή επαναληπτικών επεμβάσεων.

Τέλος, μελετήθηκε η επίδραση της τροποποιημένης ατμόσφαιρας κατά τη συσκευασία στη μετασυλλεκτική συμπεριφορά των ποικιλιών μεσπιλιάς ‘Καραντώκη’ και ΄Μορφίτικη’. Ο καρπός χαρακτηρίζεται από σχετική μικρή διατηρησιμότητα κατά τη διάρκεια της μετασυλλεκτικής τους διαχείρισης, εν μέρει λόγω διαταραχών κρυοτραυματισμού. Σε αυτή τη μελέτη αξιολογήθηκε η εμπορική συσκευασία Xtend® σε καρπούς που υποβλήθηκαν σε ψυχρή συντήρηση, σε συμβατικό ψυγείο (4°C), για 3 εβδομάδες και στη συνέχεια διατηρήθηκαν σε θερμοκρασία δωματίου για 0, 2, 4 και 7 ημέρες, αντίστοιχα. Τα αποτελέσματα ανέδειξαν τα οφέλη της συσκευασίας τροποποιημένης ατμόσφαιρας όσον αφορά την μετασυλλεκτική εμφάνιση του καρπού ακόμη και μετά την παρατεταμένη διατήρησή τους, υπό την προϋπόθεση ότι οι καρποί θα καταναλώνονται σε σύντομο χρονικό διάστημα μετά την έξοδό τους από την ψυχρή

συντήρηση. Επιπλέον, αναδείχτηκε η υπεροχή της ποικιλίας ‘Μορφίτικη’ σε σχέση με την ποικιλία ‘Καραντώκη’ αφού οι καρποί της πρώτης παρουσίασαν αισθητά καλύτερα φαινοτυπικά αποτελέσματα μετά τη μετασυλλεκτική διατήρηση τους.

Η παρούσα διδακτορική διατριβή παρέχει νέα πληροφόρηση στην επίδραση προσυλλεκτικών και μετασυλλεκτικών χειρισμών, στην ποιότητα και τα φυτοχημικά χαρακτηριστικά των καρπών μεσπιλιάς, μιας καλλιέργειας που έχει μελετηθεί ελάχιστα παρά την οικονομική της σημασία. Σημαντική σύνδεση διαφάνηκε μεταξύ της υψηλής περιεκτικότητας καροτενοειδών σε ιστούς (32 στην επιδερμίδα και 18 στην σάρκα) και της έκφρασης των γονιδίων που μελετήθηκαν κατά την διάρκεια των αναπτυξιακών σταδίων του καρπού. Λαμβάνοντας υπόψη την οικονομική σημασία του φαινομένου του ιώδους μεταχρωματισμού (το οποίο επηρεάζει αρνητικά την εμπορευσιμότητα της καλλιέργειας) διαπιστώθηκε συσχέτιση μεταξύ του καταβολισμού της σουκρόζης και της εμφάνισης του ιώδους μεταχρωματισμού σε καρπούς μεσπιλιάς των τριών ποικιλιών ‘Καραντώκη’, ΄Μορφίτικη’ και ‘Obusa’ με διαφορετικό βαθμό εμφάνισης συμπτωμάτων. Παρατηρήθηκε ψηλότερος καταβολισμός της σουκρόζης σε γλυκόζη στην επιδερμίδα των καρπών με μεγαλύτερη εμφάνιση συμπτωμάτων ιώδους μεταχρωματισμού και αναδείχτηκε ο πιθανός ρόλος της έκφρασης των γονιδίων *CWI3* και *VI* που επηρεάζουν αυτή τη διαταραχή, με πιθανή χρήση σε προγράμματα γενετικής βελτίωσης. Επιπλέον, αναδείχτηκε η σημασία του γονότυπου της γηγενής ποικιλίας μεσπίλιας ΄Μορφίτικη’, όσον αφορά την περιεκτικότητα των καρπών της στα δεσμευμένα φαινολικά συστατικά τα οποία συμβάλλουν σημαντικά στο συνολικό φαινολικό περιεχόμενο του καρπού. Με σκοπό να βελτιωθούν τα μετασυλλεκτικά ποιοτικά χαρακτηριστικά του καρπού μεσπιλιάς έγιναν δοκιμές μέσω διαφυλλικών ψεκασμών. Τα αποτελέσματα έδειξαν ότι ο διαφυλλικός ψεκασμός με ακετυλοσαλικυλικό οξύ είναι μια υποσχόμενη και οικονομικά συμφέρουσα εφαρμογή η οποία θα πρέπει να μελετηθεί περαιτέρω για παραγωγή ανώτερης ποιότητας καρπών μεσπιλιάς. Σε τεχνολογικό επίπεδο και λόγω της μικρής διατηρησιμότητας του καρπού μετασυλλεκτικά, μελετήθηκε η συσκευασία τροποποιημένης ατμόσφαιρας (MAP) η οποία παρέχει τη δυνατότητα επέκταση της εμπορικής ζωής καρπών μεσπιλιάς μέσω ψυχρής συντήρησης.

Λέξεις-κλειδιά: *Eriobotrya japonica*, αναπτυξιακά στάδια, ωρίμανση καρπού, β-καροτένειο, β-κρυπτοξανθήνη, λουτεΐνη, βιοσυνθετικό μονοπάτι των καροτενοειδών, ψυχρή συντήρηση, φυτοχημικά, ελευθέρα φυτοχημικά, δεσμευμένα φυτοχημικά αντιοξειδωτική ικανότητα, ιώδης μεταχρωματισμός, φυσιολογική διαταραχή, μετασυλλεκτική διαταραχή, έκφραση γονιδίων, σουκρόζη, γλυκόζη, φρουκτόζη, φαινολικές ενώσεις.

ABSTRACT

Loquat (*Eriobotrya japonica* Lindl.) belongs in the Rosaceae family and Pomoideae subfamily, originated from southwest China. It is a subtropical evergreen fruit tree that blooms in fall and early winter. Its unique reversed annual cycle enables the species to adapt well in the Mediterranean basin and other subtropical climates. Over the recent years the worldwide loquat cultivation has been intensified due to the excellent organoleptic characteristics of the fruit, high nutritional value as well as reduced phytosanitary problems. In Cyprus, the loquat cultivation is mainly favoured in the coastal regions, covered by mesh and/or plastic. 'Karantoki' and 'Morphitiki' are the two predominant cultivars. The production is exclusively destined for domestic consumption due to low production volumes that are being produced and the high perishability of the commodity.

Loquat commercial production has risen in interest as it has been shifted from a small local cultivation to a niche product that nowadays can gain added value as it is available during late winter- early spring period. In view of the abovementioned actualities, the current dissertation was composed of five independent yet interrelated experiments. The first two fundamental studies dealt with: (1) the analytical and molecular carotenoid profile investigation of loquat fruit during successive on-tree developmental stages and (2) the involvement of polyphenolic (*PAL1*, *PAL2* and *PPO1*) and carbohydrate (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) biosynthesis transcripts in the incidence of purple spot physiological disorder. In addition, three studies with technological perspectives were employed: (1) the effect of harvesting day on phytochemical attributes, mechanical properties and postharvest performance of loquat fruit, (2) the effect of foliar spray applications [acetylsalicylic acid (ASA), spermidine (Spd), and a commercial calcium supplement] on postharvest performance of loquat fruit and (3) the effect of modified atmosphere packaging on qualitative attributes of loquat fruit after refrigerated storage.

Initially, the metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway was examined and revealed distinct regulatory patterns between the peel and the flesh tissue of loquat fruit cv. 'Obusa' during successive on-tree developmental stages. A link between gene up- or down- regulation during the developmental stages of the loquat fruit, and how their expression affects carotenoid

content per tissue (peel or flesh) was established. Substantial changes regarding colour parameters, both between the peel and the flesh and among the different developmental stages, were monitored, concomitant with a significant increment in carotenoid content with the progress of on-tree development. Key genes and individual compounds that are implicated in the carotenoid biosynthetic pathway were further dissected with the employment of molecular (RT-qPCR) and advanced analytical techniques (LC-MS). Results revealed significant differences in carotenoid composition between the peel and the flesh. Thirty-two carotenoids were found in the peel, while only eighteen carotenoids were identified in the flesh**.** The major carotenoids in the peel were *trans*-lutein and *trans*-β-carotene; the content of the former decreased with the progress of ripening, while the latter registered a 7.2-fold increase. However, carotenoid profiling of loquat flesh indicated *trans*-β-cryptoxanthin, followed by *trans*-β-carotene and 8-epoxy-β-carotene to be the most predominant carotenoids. High amounts of *trans*-lutein and *trans*-β-carotene were supported by significant induction in *BCH* and *CYCB* gene expression, respectively, while lower amounts of *trans*-lutein in the flesh compared with the peel was correlated with significant suppression of *LCYB* and *LCYE* expression levels.

Considering that loquat market potential is severely affected by purple spot (PS), a pre-harvest physiological disorder, evident as skin discoloration with depressed purple surface, the intensity and severity of this disorder in three loquat cultivars (‘Morphitiki’, ‘Karantoki’ and ‘Obusa’), with different degree of sensitivity, was monitored. At commercial maturity stage, harvested fruit were segregated into five distinct groups based on the severity incidence of PS. ‘Obusa’ fruits showed the highest incidence of affected fruit (58.6 %), while those harvested from ‘Morphitiki’ did not show any purple spot symptoms. ‘Karantoki’, showed an intermediate severity, with 31.3 % of the fruit being affected by the disorder. Higher total soluble sugars (TSS), sucrose (Suc), glucose (Glu) and fructose (Fru) contents were exhibited in cv. ‘Karantoki’ peel tissue with PS. The lower TSS, Glu and Fru contents were registered for asymptomatic cv. ‘Obusa’ peel tissue, whereas lower content of Suc was monitored in cv. ‘Obusa’ with PS. Furthermore the role of genes implicated in the chlorogenic acid (*PAL1, PAL2, C3H* and *PPO1*) and sucrose (*CWI2, CWI3, SPS, SPS1, SPS2, NI2, NI3, SUSY, HXK, FRK* and *VI*) biosynthetic pathways were dissected. Results indicated that the gene

expression of *CWI3* and *VI* was highly correlated with the incidence of PS appearance in ‘Obusa’ and can potentially be used as markers in loquat breeding programs.

Subsequently, the effect of harvesting day on mechanical properties, postharvest performance and phytochemical attributes (free and bound phenolic compounds and antioxidant capacity) of the predominant loquat cultivars grown in Cyprus (cvs. ‘Karantoki’ and ‘Morphitiki’) was examined. Determination of the aforementioned attributes at harvest (H) and after additional maintenance at room temperature for 3 days (H+3) for four successive harvesting dates (H1-4) were determined. Flesh firmness was slightly higher in early-harvested compared to late-harvested fruit, while slight or no differences after shelf life period for both cultivars were monitored. ‘Karantoki’ fruits manifested higher values of ripening index (SSC/TA) than ‘Morphitiki’; such values were higher with the progress of harvest date due to a significant decrease of titratable acidity. No evident differences were registered between the harvest date and the shelf life period for both cultivars regarding free phenolic content. Results highlighted the significance of bound phenolics that contributed to the phenolic fraction of loquat fruit for 21.6-37.5%, depending on the cultivar and storage condition applied. This study additionally provides new information in the unexploited area of bound phytochemical properties of loquat fruits. ‘Morphitiki’ fruits were generally characterized by higher bound phenolic content, along with higher DPPH antioxidant capacity, compared to ‘Karantoki’ fruits that exhibited higher FRAP antioxidant activity.

Thereafter, the effect of foliar spray applications with acetylsalysilic acid (ASA), spermidine (Spd), a combination of the two (ASA + Spd) and a commercial calcium product was applied in order to investigate their effect on yield efficiency, qualitative attributes, textural properties and the incidence of physiological disorders, namely purple spot and fruit cracking. Acetylsalicylic acid is recommended as a cost-effective treatment that needs to be further explored towards production of loquat fruit with enhanced properties. However, spermidine application alone or in combination with acetylsalicylic acid did not show any beneficial and/or synergistic effect on loquat fruit quality parameters to justify its application. Foliar applications did not affect fruit growth and yield efficiency. The current study shed some light on the potential use of foliar spraying with acetylsalicylic acid, alone or potentially in combination with a calcium supplement, towards enhancement of fruit quality properties of an added-value product as loquat. To this aim, further studies under different environmental conditions

that may accelerate the incidence of physiological disorders and/or multiple applications over the growing season need to be implemented.

Finally, the effect of modified atmosphere packaging (MAP) on postharvest performance of the two most commercially important loquat cultivars grown in Cyprus (cvs. 'Karantoki' and 'Morphitiki') was evaluated. Loquat fruit is characterised by relatively short storage potential partially due to chilling-related disorders. In this study, the evaluation of Xtend® packaging on fruit that was subjected to cold storage, in conventional refrigerator (4°C), for 3 weeks and subsequently allowed at room temperature for 0, 2, 4 and 7 days was examined. Results indicated the beneficial effect of the commercial MAP in maintaining postharvest appearance of loquat fruit, even under extended refrigerated storage, provided that fruit will be consumed in a short period after removal from refrigerated storage. Furthermore, 'Morphitiki' fruits presented appreciably better postharvest appearance compared to 'Karantoki fruit, highlighting the genotype significance.

This study provides new knowledge in the effect of pre- and post-harvest handling, on the quality and phytochemical characteristics of loquat fruit, a poorly analysed crop compared to its economic importance. An important link was established between the high carotenoid content per tissue (peel or flesh) with the expression of the identified genes responsible for this expression (32 in peel and 18 in flesh) during on tree developmental stages. Considering the economic importance of loquat fruit and in order to shed some light on the incidence of PS disorder (that severely affects the marketability of the crop) our study revealed that PS is genotype-dependent and associated to sugar catabolism as 'Obusa', 'Karantoki' and 'Morphitiki' fruits exhibited severe, intermediate or no PS symptoms, respectively. As higher catabolism of sucrose to glucose is noted in loquat fruit peel with PS. The potential use of *CWI3* and *VI* genes as molecular markers in the detection of PS was proposed, as the two genes were highly correlated with the sugar catabolism and the PS appearance. The genotype significance of the indigenous loquat cultivars 'Morphitiki' and 'Karantoki', was studied in terms of phenolic content and antioxidant capacity. The bound phenolics importance was established as they significantly contribute to the total phenolic fraction of loquat fruit. With the aim to enhance qualitative attributes of loquat fruit and to regulate physiological disorders by applying preharvest foliar application treatments, results

showed that ASA can be recommended as a cost-effective treatment that needs to be further explored towards production of loquat fruit with enhanced properties. Due to the fact that loquat fruit is characterised by relatively short storage potential, the current study reported the beneficial effect of a commercial MAP in extending the marketability of the loquat fruit for up to 3 weeks in a refrigerated cold storage.

Keywords: *Eriobotrya japonica*, developmental stages, maturation, ripening, β-carotene, β-cryptoxanthin, lutein, LC-MS, carotenoid biosynthetic pathway, cold storage,chilling, phytochemicals, free phenolics, bound phenolics, antioxidant capacity, purple spot, preharvest disorder, gene expression, sucrose, glucose, fructose, phenolic compound

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§	Section
°C	Celsius
BC	Before Christ
ca.	Approximately
CO_2	Carbon dioxide
cv.	Cultivar
Etc	Etcetera – and other similar things
Fe	Iron
Zn	Zinc
g	Gram
Ha	Hectare
HPLC	High Performance Liquid Chromatography
i.e.	In other words
Kg	Kilogram
L	Liter
LC	Liquid chromatography
LSD	Least significant difference
ABA	Abscisic acid
Chl a	Chlorophyll-a
Chl b	Chlorophyll-b
FW	Fresh weight
EjACT	*Eriobotrya japonica Actin*

DXS	*1-deoxy-D-xylulose 5-phosphate-synthase*
DXR	*DXP reductoisomerase*
PSY1	*Phytoene synthase*
PDS	*Phytoene desaturase*
ZDS	*f-carotene desaturase*
CRTISO	*Carotene isomerase*
LCYB	*Lycopene β-cyclase*
CYCB	*Chromoplast-specific lycopene β-cyclase*
LCYE	*Lycopene ε-cyclase*
BCH	*β-carotene hydroxylase*
ECH	*ε-carotene hydroxylase*
ZEP	*Zeaxanthin epoxidase*
VDE	*Violaxanthin de-epoxidase*
IDS	Isopentenyl pyrophosphate synthase;
IDI	Isopentenyl pyrophosphate isomerase
GGPS	*Geranylgeranyl diphosphate synthase*
M	Meter
m/z	Mass-to-charge ratio
mg	Miligrams
mL	Milliliter
mM	Millimole

mm	Milimeter
Mol	Mole
MS	Mass spectrometry
Nm	Nanometers
O_2	Oxygen
ZISO	*ζ-carotene isomerase*
NSY	*Neoxanthin synthase*
NCED	*9-cis-epoxycarotenoid dioxygenase*
GAP	D-glyceraldehyde 3-phosphate
HMBPP	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate
DMAPP	Dimethylallyl pyrophosphate
IPP	Isopentenyl pyrophosphate
GGPP	Geranylgeranyl diphosphate
UPLC	Ultra performance liquid chromatography
v/v	Volume to volume
w/v	Weight to volume
WL	Water loss
Mm	Micrometer
SSC	Soluble solids content
TA	Titratable acidity
H	Harvest day
H+3	Harvest plus three days shelf life

H+6	Harvest plus six days shelf life
RI	Ripening Index
FF	Flesh Firmness
WL	Weight Loss
GAE	Gallic acid equivalents
PAs	Phenolic Acids
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TE	Trolox equivalent
FP	Free phenolic content
BP	Bound phenolic content
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
PS	Purple Spot
SI	Severity Incidence
SSC	Soluble Solids Content
Suc	Sucrose
Fru	Fructose
Glu	Glucose
SE	Standard error
PA	*Phenylalanine ammonia lyase*
C3H	*p-coumarate 3-hydroxylase*
PPO	*Polyphenol oxidase*

AI	*Acid invertase*
CWI	*Cell wall invertase*
SPS	*Sucrose phosphate synthase*
SPP	*Sucrose phosphate phosphatase*
A/NI	*Alkaline/neutral invertase*
CIN	*Cytoplasmic invertase*
SUSY	*Sucrose synthase*
HXK	*Hexokinase*
FRK	*Fructokinase*
VI	*Vascular invertase*

1 Introduction

1.1 Origin and botanical characteristics

The loquat (*Eriobotrya japonica* Lindl.), also known as Japanese plum tree or the yellow plum tree, belongs to the Rosaceae family and Pomoideae subfamily, originated from southwest China. It is a subtropical evergreen fruit tree that blooms in fall and early winter period (Lin et al., 1999). The cultivation of loquat in Japan was introduced from China in ancient times (**Figure 1.1**) and the first descriptions of the fruit in Japan were found to be as early as 1180 (Badenes et al., 2009). People beyond eastern Asia first learned of the loquat from the German traveller and physician Englebert Kaempfer, who observed it in Japan and described it in Amoenites Exotica in 1712, while the Swedish botanist, Carl Peter Thunberg, in Flora Japonica (1784), described it for the first time as a species and included it in the *Mespilus* genus (Lin et al., 1999; Badenes et al., 2009). In 1784, the loquat was introduced from Guangdong into the National Garden at Paris, and in 1787 it was introduced into the Royal Botanical Gardens at Kew, England. Thereafter, loquat was distributed around the Mediterranean countries, including Algeria, Cyprus, Egypt, Greece, Israel, Italy, Spain, Tunisia and Turkey. Sometime between 1867 and 1870, loquat was introduced to Florida from Europe, and to California from Japan. Chinese immigrants are assumed to have transferred the loquat to Hawaii (Morton, 1987). Cultivation was then spread to India and South-eastern Asia, the East Indies, Australia, New Zealand, Madagascar, and South Africa (**Figure 1.1**). Loquats are now distributed in many Asian countries, for example, Laos, Nepal, Pakistan, South Korea, and Vietnam, in Armenia, Azerbaiian and Georgia as well as in the Americas, including Argentina, Brazil, Chile, the mountains of Ecuador, Guatemala, Mexico, and Venezuela (Badenes et al., 2009).

In 1822, the English botanist, John Lindley, named the genus *Eriobotrya* from Greek word, *erio-*, wool, and *botrys*, a cluster, referring to the woolly, clustered panicles. *Eriobotrya* genus is often confused with *Mespilus* genus, and sometimes with *Crataegus* or *Photinia*. The number of loquat species is disputed and opinions vary among authors. However, it is known that there are at least 22 species and 10 varieties or *forma*. Most of them are originated in southern China, and the remainder in Southeastern Asia (Lin et al., 2007).

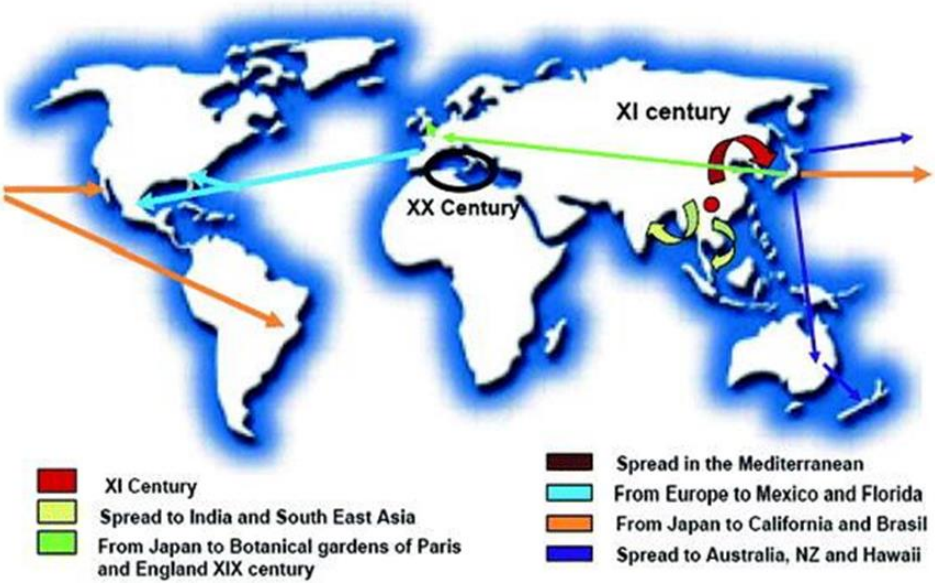

Figure 1.1: Origin and spread of loquat species (Source: Badenes et al., 2009).

The taxonomy of genus *Eriobotrya* is also still under dispute. One system is based on the lower surface of young leaves having hair or not, and another system is based on blooming from late autumn to early winter, or spring, but neither is accepted widely. Only *Eriobotrya japonica* is cultivated for its fruit; *Eriobotyra deflexa* and *E. prinoides* have been used as rootstocks, but they are less widely used than *Photinia serrulata* Lindl. in China and *Cydonia, Malus, Pyrus* and *Pyracantha* in Mediterranean regions (Badenes et al., 2009).

The loquat fruits are spread worldwide, as observed in various regions of Asia continent (Japan, India, Madagascar and South Korea), Mediterranean countries (Spain, France, Turkey, Greece, Cyprus, Portugal and Italy) and across the Americas (United States, Brazil, Argentina and Chile) (Lin et al., 1999; Caballero and Fernandez, 2004; Lin et al., 2007; Badenes et al., 2009; Gong et al., 2015). Loquat commercial production has risen in interest as it has stepped out from a small local cultivation to a niche product that enjoys appreciably high prices, particularly the early harvested fruit (Goulas et al., 2014), mainly due to the lack of other fleshy fruits into the market during its harvesting period (February-May).

The species can reach up to 10 m in height, but in areas of intensive cultivation the growth is generally smaller, about 3-4 m. The canopy is rounded; the branches are

velvety and composed of simple, alternate leaves, generally with elliptic-lanceolate format of rigid texture and serrated border with size ranging from 10 to 25 cm and a very intense coloration in dark green tone on the upper face that is usually lustrous, whereas the underside is white or rusty (**Figure 1.2 A**).

The loquat tree has three flushes of growth per year and the principal tree growth can be separated into 8 distinct developmental stages (Martinez-Calvo et al., 1999) namely 'bud development' [growth stages (010-039)], 'leaf development' [growth stages (110-139)], 'shoot development' [growth stages (311-339)], 'inflorescence emerge' [growth stages (500-509)], 'flowering' [growth stages (600-609)], 'fruit development' [growth stages (701-709)] and finally 'maturity of fruit' [growth stages (801-809)]. In particular, under Mediterranean weather conditions, the tree blooms between October and early November and its fruit develops through winter, reaching the maturation stage from early February until May, depending on the cultivation practices and the cultivar considered. The flowers are about 2 cm in diameter, white, sweetly fragrant, with four petals and produced in brunches with three to ten flowers and, before opening, have a velvety texture (Lin et al., 1999; Delucchi and Keller, 2010). The flowers are extremely rich in nectar and can be used in honey production especially since there are not many nectar producing flowers during the autumn period (**Figure 1.2 B**).

Figure 1.2: *Eriobotrya japonica* leaf with distinct dark green colour on the top and white underside (A), bee collecting nectar from loquat flowers (B).

Loquat fruits are characterised of the pommel type, with the fleshy portion composing a small floral container (3-5 cm diameter) and a shape varying from spherical to oval and a unit weight of about 10-80 g although there are reports of weight up to 170 g (Lin et al., 1999). The peel has a smooth texture and a yellow- orange and sometimes pink color (Pio et al., 2007a.b). The flesh is juicy, presenting a pleasant aroma with a coloration ranging from white to orange- salmon (**Figure 1.3**). However, segregation of loquat cultivars based on their flesh color can be confusing, since additionally the terms yellow- and orange-fleshed are being used. White-fleshed cultivars have a creamy, pale yellow color, while the terms red- and orange-fleshed can be considered as synonymous. The seeds are located in the center of the fruits, and often found in number of 3-5 per fruit with brown coloration (**Figure 1.3)**. The trunk is relatively short and the root system is characterized as superficial, extending approximately 25-30 cm deep (Ojima et al., 1999; Delucchi and Keller, 2010; Rodriguez, 2018).

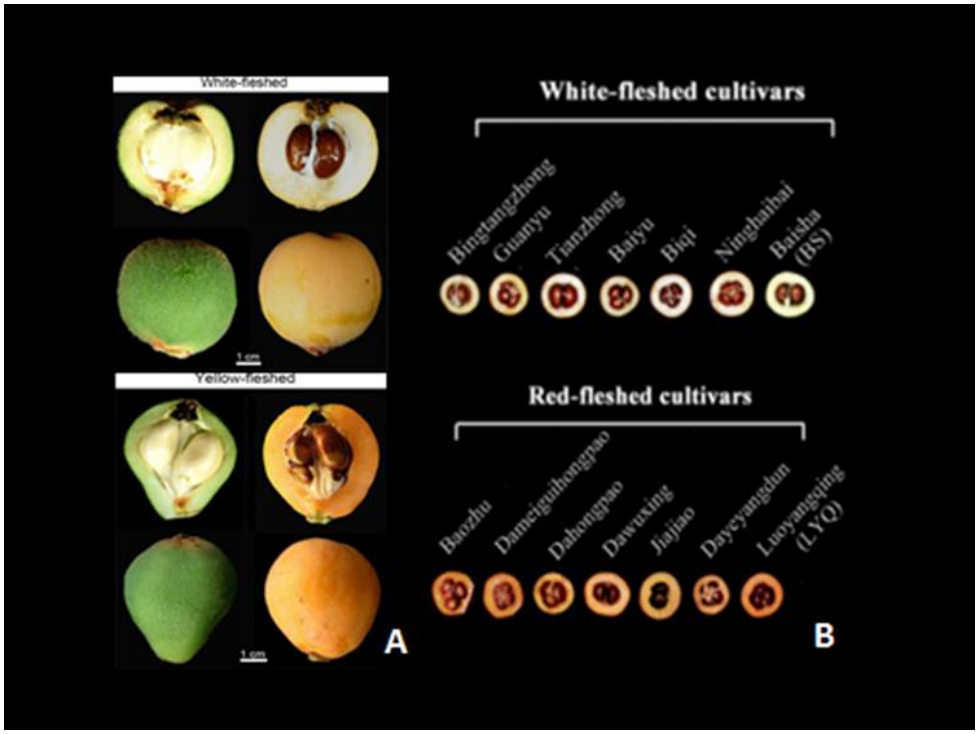

Figure 1.3: Caption of white and yellow fleshed cultivars (A; Source: Cai et al., 2019) and white and red-fleshed cultivars (B; Source: Fu et al., 2014).

1.2 Field Cultivation

1.2.1 Climate adaptation and soil requirements

The loquat tree has been adapted in a subtropical climate with mild temperatures. Generally loquat cultivation is spread between latitudes 20 and 35° North or South, but closer to the sea it can be cultivated up to 45° latitude (Lin et al., 1999). Extreme summer temperature is decisive in fruiting and dry hot winds cause leaf burns (**Figure 1.4**). Cold winter climates or in extreme cold and wet climates the tree can grow as an ornamental plant and bears no fruit.The actual tree can withstand temperatures of over -12°C. On the other hand, small fruit that is the most durable [BBCH stage 701 (Martinez-Calvo et al., 1999)], can only survive with temperatures over -3°C. Lower temperatures can cause seed distraction, fruit cracking as well as fruit fall. Flowers can also be damaged by low temperatures in autumn. Fruit fall can also occur when sustained high temperatures. Lin et al. (1999) mentioned that winter temperature should be higher than -3°C and under 35°C in the summer. De Almeida Lopes et al. (2018) notes that loquat adapts well to temperate and subtropical regions with average annual temperature of 15°C and altitude over 600 m and annual rainfall of 1200-1800 mm.

Figure 1.4: Leaf and shoot burning due to extreme temperatures and saline water.

The loquat tree is not very demanding in terms of soil needs. It has normal growth in a range of medium fertility soils from light clay to heavy clay. Optimum production is achieved when planted in medium fertility soils. In order to avoid rotting, the soil needs to be well drained. It has also been observed that the tree is sensitive to saline soils as well as brackish water, and as leaf and shoot burning may occur under such adverse conditions (**Figure 1.4**). Other studies also mentioned that soil should be deep and well drained, with an adequate content of organic matter (De Almeida Lopes et al., 2018). Sand loams or clay loams with pH of 5.0 to 8.0 are considered appropriate with an optimum pH of 6.0 (Lin et al., 1999; De Almeida Lopes et al., 2018).

1.2.2 Cultivation practices

Propagation: Traditional propagation by seed, in order to produce rootstock, has been the most common method in many loquat producing countries. This method cannot be widely recommended because of the genetic segregation of the produced plants. Seed propagation is mainly favored when the trees are for ornamental use. The seeds have to be planted immediately as they do not remain viable for long after their removal from the fruit. *Eriobotrya japonica* as well as *Eriobotrya germanica* seedlings on the other hand are often used as rootstock. Quince rootstock can also be used but it has been noted to cause dwarfing (Lin et al., 1999) that might not be desirable in open field loquat cultivations. In the Mediterranean countries, seedlings of *Eriobotrya japonica* are preferred as rootstock as they are well adapted to calcareous soils often found in the Mediterranean region. Quince has been studied often as a rootstock as it enhances the fruit size, it provides higher sugar accumulation and better fruit coloration. Loquat trees grafted on quince are smaller, more compact (De Almeida Lopes et al., 2018), have shorter juvenile period but are very sensitive to calcareous soils and often show incompatibility with many cultivars (Llacer et al., 2003). Grafting is a common method of variety propagation in loquat breeding, the slow growth of rootstocks is a main factor limiting the expansion of this technique. Phosphorus nutrition could significantly promote the occurrence of fibrous roots, while excessive phosphorus supply might disturb the absorption and utilization of nitrogen of roots, intensify the

lignification process of the main stem, and then affect the growth of the aboveground part (Xu et al., 2020).

Chip, patch, cleft and T (shield) buddings are used for grafting the desirable cultivar on rootstock (Lin et al., 1999; De Almeida Lopes et al., 2018). Air layering is also used in China by many farmers. Cuttings dipped in indole-3-butyric acid (IBA) solution under mist installation are also used in Japan. Similarly in Egypt cuttings dipped in 4.000 ppm IBA solutions and then placed in a sand:peat moss medium (1:1) under mist with satisfactory rooting results (Lin et al., 1999).

Under Cypriot conditions, loquat tree that has been grafted will have an adequate yield in five years whereas seedlings will start producing fruit after the eighth year. Seedlings over the age 10 can be grafted after strict pruning

Orchard Planting: Preferably loquats are planted at a density of about 500-600 trees/ha (4 to 4.5 m between rows and 4 to 4.5 m between trees) but when there are vigorous cultivars the density can reduce to 450 trees/ha (5 x 4 m). In Japan, the standard planting distances are 5 to 7 m, whereas in Brazil 7 x 7 m is recommended on flat land, and 8 x 5 (or 6) m on the slopes (Lin et al., 1999). When the trees are grafted on quince, the planting distances are smaller (5 x 3 and 4 x 2 m) as the specific rootstock cause dwarfing (Pio et al., 2008). Planning windbreaks in order to avoid fruit damage from cold or warm winds as well as branch breaking is essential especially in areas with strong winds.

Pruning: The preferred pruning system is an open-center system as this allows better load distribution, better lighting and airing as well as all necessary spraying, thinning and pruning are performed with ease. Pruning after the initial tree training should be light in order to promote new vegetation.

Thinning: The fruit thinning is necessary practice in loquat cultivation. This is performed by hand, soon after the cold damage period is over in order to ensure that the larger without flaws fruits are selected. Normal practice is to choose 1-5 fruits depending on the shoots robust (Pio et al., 2008; **Figure 1.5**).

In Japan, flower bud thinning is aimed at limiting the number of flowers within an inflorescence, encouraging the growth of fruitlets, and shaping the inflorescence for

easier bagging. The usual procedure of flower thinning is to remove the lower 2-3 peduncles and some upper peduncles on the inflorescence leaving the middle tree to four peduncles. Successive thinning (one to three times) is required as flower buds appear over a period of time. In China, flower thinning is rare, as farmers are not comfortable with the reduced production that flower thinning provides (Lin et al., 1999).

Figure 1.5: Before (A) and after (B) fruit thinning.

Irrigation: In general, loquat does not require irrigation but during fruit maturation, sprinkler irrigation is carried out to alleviate the detrimental effect of sunburn. In areas with not adequate rainfall the crop is irrigated and its total irrigation water requirements are around 5000-6000 m^3 / ha. The tree is very sensitive to salts, so the water used must have low salinity and definitely low boron content.

Fertilization: Orchards are usually mowed 2-3 times per year and mowed grass clippings are spread under the trees as mulch. The soil can be improved by incorporating manure and other organic substrates. This provides the soil with extra moisture, better nutrient status and soil bulk. It also improves the nitrogen (N), phosphorous (P), potassium (K) nutrients thus increase total yield (Lin et al., 1999). During the first year, 60 g of nitrogen fertilization should be applied around the seedling in four doses of 15 g. From the second year the recommended dose increases from 60 – 120 g per tree of the N, P, K nutrients. From the fourth year onwards the production

fertilization is 60 – 120 kg/ha of N, 20-90 kg/ha of P and 20 – 100 kg/ha of K (Pio et al., 2006; 2008).

Loquat fertilization, in the Mediterranean region, begins end of September and continues until beginning of March. This is due to the unique production cycle or the tree as it is flowering in autumn and bears fruit in spring. The main deficiencies that are observed in loquat cultivation are iron (Fe) and zinc (Zn). The Fe deficiency causes leaf discoloration with a distinct yellow colour with the shouts to exhibit small leaves and fruit. On the other hand, Zn deficiency appears with smaller top leaves and the broom effect at the top of the shouts. The symptoms can be dealt with soil or spraying fertilizing.

Harvest: Fruit yield is normally harvested in 2-3 consecutive hands with 15 - 20 days interval due to differentiation in flower budding. The fruits are easily injured so their handling should be done by experienced personnel. The fruits are difficult to harvest because of the thick, tough stalk that does not easily separate from the fruit. Individual fruits are harvested from the clusters with the aid of clippers (**Figure 1.6**).

Figure 1.6: Fruit harvesting with the aid of clippers.

Whole clusters are not considered attractive for the market with the exception of Spain that might sell the loquat fruit in clusters. Different countries have different sorting grades. In India there are usually two and occasionally three grades sold, in Japan three to four grades and in China two to three (Lin et al., 1999). Harvesting and packing is a highly intensive procedure therefore cultivation is limited per producer.

1.3 Worldwide loquat cultivation

Loquat species exist as diverse genetic lines, and germplasm collections have been established in China, Japan and Spain (Zheng, 2007; Lin, 2004; Llácer et al., 2004; Terai, 2002). Species from China possess great diversity; more than any other location. *E. japonica* has been grown in 10 different regions on China, and there are more than 1000 accessions described in several Chinese germplasm collections (Zheng, 2007). The largest collection of germplasm with more than 250 cultivars, is located in Fuzhou, however less than 50 cultivars are widely cultivated in China (Badenes et al., 2009). There are no Food and Agricultural Organization of the United Nations (FAO) statistics available for world loquat fruit production; however, China is the world's largest producer of loquat with a cultivation area of 170,000 ha, producing more than 400,000 t of fresh fruits per year (Zhang et al., 2015).

Loquat cultivars have been classified as either 'white' or 'orange' in China according to the flesh coloration, the former make up 30 % of the number of total cultivars in China (Ding et al., 1995). Some of the white flesh cultivars, such as 'Zhaozhong' and 'Baiyu', are the leading cultivars in Jiangsu province (**Figure 1.3**). Loquats have formed different ecological types in various zones during the long course of their cultivation and acclimatization. Ecotypes in China can be divided into two cultivar groups: the north subtropical cultivar group and the south subtropical cultivar group (Ding et al., 1995). There are two types of loquat that can be distinct from a botanical point of view. The first on is the 'Chinese' type that is characterised by large fruits with pear shaped and yellow fleshed fruit and the second is the 'Japanese' type that is characterised by smaller fruits that are round shaped and white or yellow fleshed (Badenes et al., 2000).

China leads internationally in terms of surface area and production (De Almeida Lopes et al., 2018; **Table 1.1**). Loquat is grown in 10 provinces with millions of trees growing secluded. Production is exclusively destined for the local market with export only reaching the 1% of the total production (**Table 1.1).** The general trend is towards increasing production with focus on the selection of more appropriate cultivars (Caballero et al., 2003). The increase is due to new technology including: genetic improvement that resulted in new cultivars such as 'Zaozhong No.6' and 'Dawuxing' (Lin et al., 2007). In 2005, 120,000 ha of loquats were planted in China with a 400,000 t production. Well known and commonly planted cultivars are mainly 'Dawuxing', 'Longqyan No 1', 'Zaozhong No 6', 'Jiefangzhong', 'Dahongpao', 'Luoyangqing', 'Ruantiaobaisha', 'Baiyu', 'Qingzhong' and 'Guanyu' (He et al., 2011).

Table 1.1: Area production and exports in the main loquat producing countries (Source: Caballero et al., 2003; Lin et al., 2007; Zhang et al., 2015).[1]

Countries	**Area (ha)**	**Production (t)**	**Exports (t)**
China	170000	400000	2000
Japan	2420	10245	
Spain	2914	41487	19400
Pakistan	11000	28800	1600
Israel	330	3000	
Egypt	33	440	
Greece	300	3000	
Morocco	385	6400	
Portugal	243	950	
Italy	663	4412	
Turkey	1470	13500	147
Chile	138		37
Brazil	300	2400	
Total	**190196**	**514634**	**23184**

[1] FAO does not provide detailed loquat production volumes and cultivation area.

Fifty-three loquat cultivars/ accessions were examined in a study that identified the genetic diversity of loquat cultivars (He et al., 2011) and are presented in **Table 1.2**, including the description of the cultivars peel and flesh color.

Japan is considered as an important loquat producing country as well as a reference region in terms of the origins of commercial varieties. The regions with the greater cultivated areas are Nagasaki and Kagosima. The production is mainly destined for the local market and the principal consumer areas are: Tokyo (47.9%), Osaka (19.9%), Yokoama (7.5%), Kyoto (4.5%) (Caballero et al., 2003). Most cultivars cultivated in Japan belong to the north subtropical group. Several cultivars, such as 'ShiroMogi', could be placed in the whitish-flesh group. Three cultivars ('Mogi', 'Tanaka', and 'Nakasakiwase) account for 95% of the total crop area (Badenes et al., 2009).

Table 1.2: Loquat cultivars/ accessions (Source: He et al., 2011).

	Cultivars/ accession	**Description**
1	Bahong	Orange-red peel, red fleshed
2	Baili	Yellow or orange-yellow peel, white fleshed
3	Baisha No. 2	White fleshed
4	Baiyu	Orange-yellow peel, white fleshed, seedling progeny of 'Zaohuang'
5	Baozhu	Yellow or orange-yellow peel, red fleshed
6	Bingtangzhong	Yellow-white peel, white fleshed
7	Biqizhong	Yellow-orange peel, white fleshed. Looks like water chestnut, seedling progeny
8	Changhong	Orange-red peel, red fleshed
9	Changlv N^{o}2	Yellow-white peel, white fleshed
10	Changlv N^{o}3	White fleshed
11	Changlv N^{o}4	Orange-yellow peel, white fleshed, large fruit derived from 'Baisha'
12	Changlv N^{o}5	White fleshed, progeny of 'Baiyu' x 'Tianzong'
13	Changlv N^{o}6	White fleshed, seedling progeny of 'Guanyu'
14	Chihong	Orange-red peel, red fleshed, seedling progeny of 'Dahongpao'
15	Chuannao	Orange-yellow peel, white fleshed
16	Daduhe	Red fleshed, small fruit, wild species, natural hybrid of *E. prinoides* and *E. japonica*
17	Dahongpao	Orange-red peel, red fleshed, deep orange-red peel and pulp
18	Dahongsha	Red fleshed
19	Dameiguihongpao	Orange-red peel, red fleshed, seedling progeny of 'Dahongpao'

20	Dawuxing	Orange-yellow peel, red fleshed, with a star like navel
21	Dayeyangdun	Orange –yellow peel, red fleshed
22	Dazhong	White fleshed
23	Dongshanjibai	White fleshed
24	Erzao	Orange –yellow peel, red fleshed
25	Gaoliangjiang	Orange –yellow peel, white fleshed
26	Guanyu	White fleshed, seedling progeny of 'Baisha'
27	Hongmao	Red fleshed
28	Jiajiao	Yellow peel, red fleshed
29	Jibanbai	Yellow peel, white fleshed
30	Jidanhong	Orange-yellow peel, red fleshed, seedling progeny
31	Jicfangzhong	Orange-red peel, red fleshed, seedling progeny of 'Dazhong'
32	Jinfeng	Orange-yellow peel, red fleshed
33	Liufenzong	Orange-yellow peel, red fleshed
34	Liye	*E. prinoides* red fleshed fruit, small fruit, wild species
35	Longquan N^{o}1	Orange-yellow peel, red fleshed
36	Luoyangqing	Orange-yellow peel, red fleshed, sepal around areas still green when ripe
37	Marc	Orange-yellow peel, red fleshed
38	Meiyu	White fleshed, seedling of 'Baisha'
39	Moriowase	Orange-red peel, red fleshed, branch mutation of 'Mogi'
40	Peluches	Orange-yellow peel, red fleshed, probably Algerie mutation
41	Qingzhong	Orange-yellow peel, white fleshed, seedling progeny of 'Baisha', early maturing fruit
42	Ruantiaobaisha	Yellow peel, white fleshed
43	Taicheng N^{o}4	Orange-red peel, red fleshed, seedling progeny
44	Tangkebairou	Orange-yellow peel, white fleshed, large fruit, mutation of 'Baisha'
45	Tianzhong	Yellow-white peel, white fleshed
46	Tongpi	Yellow peel, white fleshed
47	Wuerbaisha	Yellow peel, white fleshed, high yield, similar to 'Ruantiaobaisha'
48	Xiangzhong	Orange-yellow peel, red fleshed with deep aroma, 'Xiangtian' x 'Jiefangzhong'
49	Xiaobaisha	Orange-yellow peel, white fleshed
50	Yingtiaobaisha	Orange-yellow peel, white fleshed, similar to 'Ruantiaobaisha'
51	Zaohuang	Orange-yellow peel, white fleshed
52	Zaohuong N^{o}6	Orange-yellow peel, red fleshed 'Jiefangzhong' x 'Moriowase'
53	Zaohuong	Yellow peel, white fleshed, seedling progeny of 'Baisha'

Spain is the second world producer of loquat with more than 40,000 t per year, and the leading exporting country with around 83% of worldwide exports, with the main

destination being EU countries: Italy, Portugal, and France (Lin et al., 2007). Spanish commercial production depends on only four cultivars: 'Algerie', 'Magdal', 'Golden Nugget' (from USA), and 'Tanaka' (from Japan) (Badenes et al., 2009) with the main production concentrating on the 'Algerie' cultivar that accounts for 90% of the total production in Spain (Besada et al., 2017).

The main area of the Spanish production is in the Alicante province, located in the Marina Baja, where Callosa d'Ensarriá, Altea, Polop and Bolulla are found in the Algar and Guadalest river valleys. The most important cultivars in the mentioned area are 'Algerie' and its mutations ('Cardona', 'Buenet', 'Cayetana', 'Amadeo'). They account for the 95 % of the total production of the area. The rest of the 5 % is produced from the varieties 'Golden', 'Magdal', 'Peluche', and 'Nadal' (**Figure 1.7**).

Figure 1.7: Main loquat cultivars in Spain (Source: Badenes).

The second area is located in Andalusia, provinces of Granada and Malaga. The cultivars grown are 'Golden Nugget' (80 % of the areas production) and 'Magdal'. There is also a small amount of 'Tanaka' production. The third area is located in the Palancia valley, which includes part of the provinces of Castellón and the Valencia and it extends from Sagunto to Segorbe. The main cultivar grown is 'Tanaka'. Other cultivars that were used but are now starting to disappear are 'Saguntí', 'Temprano de Petrés' and 'Temprano de Torres' (Caballero et al., 2003).

A germplasm collection belonging to the IVIA (Moncada, Valencia, Spain) includes 34 cultivars that originated from all over the world (Badenes et al., 2000; **Table 1.3**).

Pakistan is one of the countries with the largest planted area and the main producer in Southern Asia (**Table 1.1**) with an increasing consumption rate. There are two distinct varietal groups. The local type cv. 'Local' that is mainly consumed in the local market as it does not normally have the necessary quality for exports. This type has a planted surface area of 10,000 ha and an estimated production of 128,000 t. The second group with 'Tanaka' as the leading producing variety with 1000 ha in production with 16,000 t yield from which the 10 % is exported to the Middle East, mainly Dubai (Caballero et al., 2003).

Table 1.3: Germplasm collection of loquat cultivars in IVIA (Source: Badenes et al., 2000).

Cultivar		**Origin**	**Cultivar**		**Origin**
1	Algerie (late clone)	Spain	18	Marc	Spain
2	Algerie	Spain	19	Moggi	Japan
3	Bianco	Italy	20	Moggi Wase	Japan
4	Betera	Spain	21	Orlanza	Italy
5	Borde	Spain	22	Ottaviani	Italy
6	Buenet	Spain	23	Peluches	Spain
7	Cardona	Spain	24	Saval	Brazil
8	Crisanto Amadeo	Spain	25	Saval-1	Spain
9	Dulce Pera	Spain	26	Saval-2	Spain
10	Golden Nugget	USA	27	Saval Moreno	Spain
11	Ismael	Spain	28	Saval Nerviado	Spain
12	Italiano	Italy	29	Saval Temprano	Spain
13	Javierin	Spain	30	Temprano Torres	Spain
14	M. Aixara	Spain	31	San Filipparo	Italy
15	Magdal	Spain	32	Tanaka	Japan
16	Magdal Blanco	Spain	33	Ullera	Spain
17	Magdal Rojo	Spain	34	Vertichiara	Italy

Israel's production is destined to the local and national markets. The orchards are located along the Mediterranean coast. Zichron Ya'acov area produces the 30 % of the loquat yield but there are also orchards near the Sea of Galilee where the fruits are

ripening earlier. The main local cultivars are ‘Akko-1’ and ‘Akko-13’ (Caballero et al., 2003; Llacer et al., 2003). From the international cultivars, ‘Tanaka’ and ‘Golden Nugget’ are the most grown. The fruit is mainly consumed fresh and there is a trend towards protecting the plantations with mesh screen (Caballero et al., 2003).

Greece’s total production is destined for the local market. There is also a great amount of trees scattered outside of organized cultivations but production is fairly steady. Greece also imports fruit from Italy and Spain. The main area of production is concentrated in the prefecture of Corinth. The total production is ca. 3000 t. Most common cultivars used are ‘Rozenon’, ‘Troulotis’ and ‘Koilarato’ (Llacer et al., 2003).

Moulouya region in the north west of the country has the 80% of the total loquat planted area of Morocco. Total production is 6400 t with a yield of 15 to 25 t/ha with the main fruit production to be harvested during April. The most commonly cultivated cultivars are ‘Musca’, ‘Navela’, ‘Mkarkeb’, ‘Tanaka’ and ‘Argelino’. Loquat is considered a fruit of economic interest and the trend is towards a growth in its cultivation (Caballero et al., 2003). Llacer et al. (2003) also mentions ‘Tanaka’, ‘Saint Michel’ and ‘Algerie’ as the most common cultivars.

Algarve and Beira Litotal regions account for the 70 % of the total loquat production of Portugal. The main commercial cultivars are ‘Argelina’, ‘Tanaka’ and ‘Golden Nugget’ (Caballero et al., 2003; Llacer et al., 2003). The indigenous cultivars ‘De Silves’, ‘De Lágrimas’, ‘Portuquesa’ and ‘Palhinha’ are also commercialized. The production area has dropped due to the difficulties in finding labor to thin fruits and harvest. It is estimated that the future trend is that there will be a drop in surface area but an increase in production volumes, due to new intensive plantations in the Algarve and the removal of the abandoned plantations (Caballero et al., 2003).

Almost all of Italy’s production is located in Sicily (90 %) and its destined for the local and national markets there are no exports in the country but on the contrary they import 12,000 to 15,000 t annually from Spain (Caballero et al., 2003; Gentile et al., 2016). The most common cultivars are ‘Nespolone di Trabia’, ‘Nespolone Bianco’, ‘Vainiglia’, ‘Sanfilippara’ and ‘Virticchiara’ (Llacer et al., 2003). In Italy the local market is not fully specialized and only a small portion of produced fruit is commercialized through a classification by cultivar, flesh colour and fruit size (Gentile et al., 2016).

In Turkey almost all the plantations are located in the South-Eastern Mediterranean region. Fruit is ripening early in springtime and it is consumed fresh. Trace amount of production is exported to countries in the Middle East, Central and Northern Europe. The main problems that Turkey is facing are the risk of frost and the land availability, due to competition with other crops namely, citrus as well as greenhouses for ornamental plants and vegetables (Caballero et al., 2003). The most common cultivars are 'Akko 13', 'Tanaka', 'Golden Nugget' and 'Hatif Çukurgöbec' (Llacer et al., 2003) with the last two cultivars and 'Sayda' used in high density plantations (3x3) under protection (Polat et al., 2005).

The total estimated production of Brazil is 2400 t, with 3.7 million boxes per year production concentrated in the Sâo Paulo area (70 %) from which the 2 million boxes come from the municipality of Mogi das Cruces. Due to its location in the southern hemisphere the harvest is sold between July and November (Caballero et al., 2003; De Almeida Lopes et al., 2018). The main volume is concentrated between the months July and September were the fruit is favored by the sweets industries for the high pectin concentrations and the lack of other fruits that can be processed. The most common cultivars grown are 'Mizuho', 'Precoce de Itaquera', 'Precoce de Campinas IAC 165-31', 'Parmogi IAC 266-17', 'Crystal Nectar IAC 866-7', 'Centenarian IAC 1567-420', 'Mizuho IAC 1567-411' and 'Mizuho IAC 167-4' (De Almeida Lopes et al., 2018).

The main loquat production area is found in the center of Chile between regions IV and VI. The most commonly planted variety is 'Golden Nugget'; although 'Tanaka' and 'Early Red' are also of interest. Almost the entire production is consumed fresh with only small amounts to be exported mainly to the United States and Europe (Caballero et al., 2003).

The cultivated area in Lebanon is ca. 750 ha with a production of 15,000 to 20,000 t per year. The loquat trees are mostly growing in the coastal areas with mild winters and commercial plantations are concentrated in the south with 65 % of the total cultivated area. A small amount of orchards are also found in Becaa area at 1200 m elevation. Loquat plantations are constituted of both local and imported accessions. Traditional known accessions are 'Baladi' and 'Saidaoui' and as accessions introduced

from Spain, France, Italy and Australia are named after their place or origin e.g. ‘Faransi’, ‘Espani’, ‘Itali’ and ‘Eustrali’ (Chalak et al., 2014).

There is limited information about other countries that are producing loquat fruits without having commercial scale plantations, including Mexico, New Zealand, Australia, United States and Uruguay (Caballero et al., 2003).

1.4 Loquat cultivation under cover

The need to grow loquat in plastic greenhouses first emerged in order to protect the trees and fruits from falling volcanic ashes in Tarumizu, a major loquat growing area in Japan, and later to protect the tree from cold injury. This system proved to be profitable because of the higher prices from earlier marketing, stable production, and the speeding of labor requirements (Lin et al., 1999).

Typical loquat cultivations in Israel and in Spain are shown in **Figure 1.8** and **Figure 1.9**. In Spain an ethylene vinyl acetate plastic film cover was placed over an orchard with ‘Angelino’ and ‘Tanaka’ loquat cultivars. The cover advanced the harvesting date by six days and increased the market value (Lin et al., 1999). In Italy protected culture became a common technique as it advances fruit ripening. In Turkey protected loquat cultivation resulted in 15-20 days advance of ripening and depending on the cultivar up to 30 days, increasing also the total yield (Polat et al., 2005).

Figure 1.8: Typical covered loquat orchard in Israel.

Figure 1.9: Typical loquat cultivation in Spain without (A) and with plastic cover (B) Source: Badenes).

Most plastic greenhouses are built of iron pipes and covered with polyvinyl chloride sheets (Lin et al., 1999); whereas it is also mentioned coverage with 300 μ-thick UV+ IR PE plastic over greenhouses, with the side height of 2.5 m and the highest point 4 m, without heating mechanisms and irrigated with drip irrigation. The plastic is covered from leaf break to flowering and is being removed after harvest (Polat et al., 2005), while in some cases is removed in July after the rainy season is over (Lin et al., 1999). Because of the high humidity during flowering, highly susceptible to gray mold (*Botrytis cinerea* Pearson) that lowers fruit set, it is advisable to apply the plastic cover after full bloom as gray mold doesn't affect the fruit. Higher temperatures accelerate the growth of young fruitlets but increase fruit maturation, which results in small fruit. Therefore, minimum night temperature should not be higher than 15 °C and maximum night temperature not higher than 25 °C. Higher temperatures just before fruit maturation may cause physiological disorders in the fruits (Lin et al., 1999).

Under Cyprus weather conditions loquat orchards are mainly covered in order to be protected from hail. Most of the time hail storms have been proven destructive not only for the fruit production but also for the trees. Plastic netting is used in this occasion to provide full cover in case of a hail storm but also improves fruit quality and quantity.

There are a number of different greenhouses suitable for the plastic netting. Preferably, the netting is placed over the orchard after fruit set (**Figure 1.10),** end of October beginning of November, and it is removed at the end of the harvesting period,

during May. Thus the greenhouses should withstand wind, rain and hail. The netting is normally placed over frameworks that are securely fastened in the soil.

Figure 1.10: Typical under netting cover loquat orchard in Cyprus.

An added advantage when netting is applied over the orchards is the protection of the fruits from the birds as well as the added fruit quality, quantity and earlier harvesting period. In some areas, plastic film is also used to enhance fruit maturity but in Cypriot weather conditions one must be weary of the possible detrimental effect of high temperatures.

1.5 Nutritional value

During on-tree ripening, fruit size increases along with an increase in sugar accumulation and a decrease of titratable acidity that renders the fruit suitable for consumption. A significant increase of total phenolic content which is notably high, during the first developmental stages, decreasing steadily and then interestingly increasing again two weeks prior harvest for cv. ‘Mogi’ and one week for cv. ‘Tanaka’; identifying the chlorogenic acid as the main contributing compound for this increase (Zhang et al., 2015). The fruits, leaves, and seeds are an excellent source of bioactive compounds such as: β-carotene and β-cryptoxanthin in higher concentrations and in lower concentrations lutein, violaxanthin, α-carotene and γ-carotene, flavonoids, vitamin C and 3- and 5-caozoylquinic acids, 5-feruloylquinic acids, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-galactoside, kaempferol-3-O-rhamnoside and kaempferol-3-O-glucoside whose action has already been recognized by the pharmacological industry in combatting diseases such as breast cancer, renal,

neurological and cardiovascular problems, diabetes, and obesity, among others (De Almeida Lopes et al., 2018). Goulas et al. (2014) quantified the phenolic content, hydroxycinnamic acid derivatives, flavonol content, carotenoid content and antioxidant campacity of the two indigenous Cypriot cultivars cvs. 'Karantoki' and 'Morphitiki' with the later exhibiting higher values.

Table 1.4: The nutritional composition of loquat fruit (Source: De Almeida Lopes et al., 2018).

Nutrients	Values/100 g flesh
Moisture	86.5 – 90.2%
Calories	47– 168 kcal
Carbohydrates	9.6 – 43.3 g
Fibers	0.8 – 1.7%
Proteins	0.43 – 1.4 g
Lipids	0.2 – 0.7 g
Ashes	0.4 – 0.5 g
Vitamin C	3.0 – 10 g
Calcium	16 – 70 g
Iron	0.28 – 1.4 g
Magnesium	9 – 13 g
Manganese	0.05 – 1.0 g
Phosphorus	20 – 126 g
Potassium	266 – 1216 g
Sodium	0.5 – 1 g

The nutritional composition of loquat at physiological stage is presented in **Table 1.4** and is characterized by high levels of nutrients, including several minerals such as calcium, iron, magnesium, manganese, phosphorus, potassium and sodium. Apart from the health benefits loquat fruit is also being highly appreciated for its light, refreshing taste (Martinez-Calvo et al., 1999; Pinillos et al., 2011).

1.6 Fruit marketability and uses

Loquat fruits are normally consumed fresh, but a small amount of loquat fruit is used for canning, jams, wine, syrup, juice and candied fruits (Lin et al., 1999; **Figure 1.11**). It is also known that the leaves, flowers and fruits are traditionally used in Chinese medicine since they are linked with health-promoting properties (Zhou et al., 2007; Ferreres et al., 2009).

Figure 1.11: Loquat fruit utilization in Spain (A) and Cyprus (B).

1.7 Loquat cultivation in Cyprus

Cyprus is an island country of 9,250 sq. Km in the Mediterranean Sea, located at the eastern part of Europe. Agriculture has always been an important sector of the Cypriot economy. Following the Turkish invasion of 1974 and the occupation of 38 % of the country's territory, including Cyprus most valuable land and water resources, an accelerated declining trend set in, which is still notable today. Also, due to the rapid growth of other sectors of the economy, agriculture today contributes less than 5 % to the gross domestic product (GDP) and about 10 % to gainful employment.[2]

[2] www.moa.gov.cy

Nevertheless, agriculture continues to be significant for the Cypriot economy as it helps to maintain the environment and a good proportion of the population in rural areas; it contributes to food security and supplies the local market, including the country's sizeable tourist industry, with good quality fresh produce, and continues to earn an important amount of foreign currency through exports, which still account for over 20 % of all domestic exports according to FAO. The principal agricultural produce are barley, potatoes, grapes, citrus, wheat and olives.

The loquat cultivation is mainly favoured in the coastal regions, covered by mesh and/or plastic. 'Karantoki' and 'Morphitiki' (**Figure 1.12**) are the two traditional cultivars. There are approximately 30 hectares of cultivated land with the two main loquat cultivars from which there is an average production of 30 tonnes per ha for the covered (under mesh or plastic) cultivars and an average of 10 tonnes per ha for the outdoor trees. The production is exclusively used for domestic consumption due to low production volumes that are being produced and the high perishability of the commodity.

Figure 1.12: Mature 'Karantoki' (A) and 'Morphitiki' (B) fruit anatomy.

Loquat fruit classification in Cyprus consists of 3 distinct commercial categories according to the Cypriot loquat fruit market according to the fruit quality and size. The group with the higher fruit quality without external blemishes is categorised as 'Extra' (over 40 g) that is sold in 5 kg boxes (**Figure 1.11**), followed by the slightly smaller fruit (30-40 g) without external blemishes that is categorised as 'A'. All the smaller fruit (under 30 g) are separated into the 'B' category. Fruit with external

blemishes like cracking (due to increased rainfall) and purple spot (PS) are considered unmarketable.

'Karantoki' is probably an Italian cultivar that was transferred to Cyprus and acclimated to the environment. It is considered as an early cultivar in Cyprus with large, pear-shaped fruits (**Figure 1.12A**), 1-4 seeds, yellow peel and rich yellow flesh. The cultivar is highly appreciated by the growers and consumers due to productivity and fruit size characteristicsthat is why it is the main cultivar in the Cypriot loquat orchards (Goulas et al., 2014). 'Karantoki' has a medium sensitivity to purple spot that is affecting a high percentage of the yearly commercial value, which vary from one year to the next. The fruit is also sensitive during handling thus the packing and transport should be contacted with great care as it bruises easily especially when overripe. Goulas et al. (2014) identified the phenolic content of 'Karantoki' fruit to 31.6 ± 1.9 mg GAE 100 g^{-1} FW. In the same study, results indicated the existence of hydroxycinnamic acid derivatives and flavonols although the later were present at relatively low contents.

'Morphitiki' is also considered as a large fruit cultivar, smaller than 'Karantoki' though, round to egg-shaped (**Figure 1.12B**). It ripens 10 to 15 days after the 'Karantoki' fruit. The peel is orange-yellow and the flesh is deep yellow and juicy. The fruit contains 1-4 seeds. It is a delicious cultivar with sweet and sour taste and thinner than 'Karantoki' peel. A characteristic of the variety is the withering of the fruit during over-ripening but the fruit can withstand transport without serious bruising. 'Morphitiki' loquat fruit exhibits high phenolic content of 39.0 ± 0.5 mg GAE 100 g^{-1} FW) and hydroxycinnamic acid derivatives with the flavonol content also following a similar pattern with total phenolics but they were present at relatively low contents. Overall 'Morphitiki' manifested higher carotenoid content than 'Karantoki' and despite its non-climacteric type, carotenoid biosynthesis increased as shelf life increased. The cultivar also demonstrated higher antioxidant capacity at all storage regimes and time points tested compared to 'Karantoki' fruit (Goulas et al., 2014).

1.8 Purple spot

The marketability of loquat is severely affected by the development of purple spot (PS), a preharvest physiological disorder that leads to skin discoloration. This disorder mainly affects the fruit surface exposed to the sun, heavily compromising

general fruit appearance and rendering them unacceptable for marketing (Gariglio et al., 2002). In the same study it was postulated that purple spot initiates at the deepest rind cells of the fruit, causing cellular dehydration and material deposition between the shrunken cytoplasm and the cell wall.

The severity of PS symptoms may substantially vary from year to year. Early harvest favors the development of PS compared with late harvest (Gariglio et al., 2003a). This is consistent with reports about the existence of environmental factors responsible for the onset of PS appearance in loquat fruit, such as minimum daily temperature and direct exposure of fruit to sun at breaker stage. However, to what extent such factors affect PS appearance still remains unknown.

A positive correlation between PS appearance and fruit flesh sugar concentration has been proposed, since fruit thinning led to higher incidence of the disorder **(**Gariglio et al., 2003b). In addition, low temperature during the night and sun exposure also increased the gradient of total sugar concentrations in favor of the flesh compared with the rind, thus increasing the incidence of PS (Gariglio et al., 2008a). A companion study from the same group showed that there is a correlation between PS occurrence on loquat fruit and the alteration of water relations between flesh and peel at the fruit color break (Gariglio et al., 2008b).

In summary, the higher PS incidence has been attributed to the simultaneous occurrence of flesh high sugar accumulation, the high growth rate enhanced by fruit thinning and environmental factors such as low night temperatures and direct sunlight exposure. There are only a few studies that deal with the PS disorder though recently new approaches were applied through hyperspectral imaging and machine learning techniques in order to identify PS among other defects in cv. ‘Algerie’ fruit (Munera et al., 2021; **Figure 1.13**).

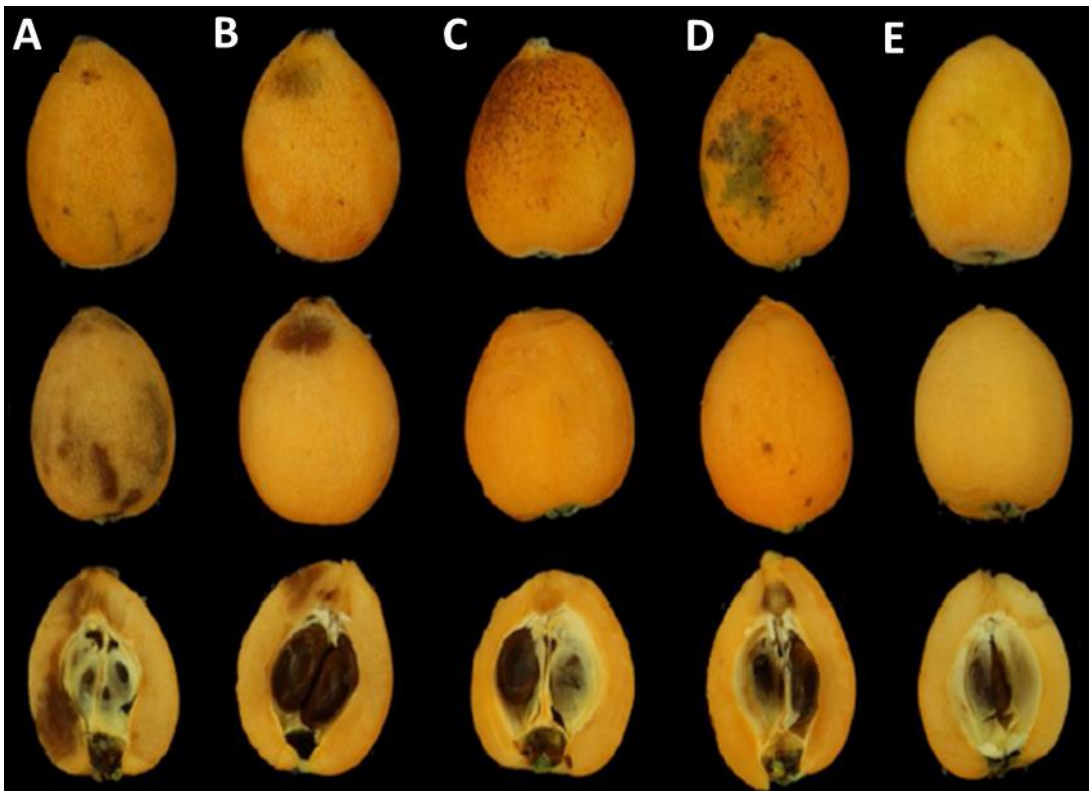

Figure 1.13: Example of common defects in 'Algerie' loquat fruit (Source: Munera et al., 2021).[3]

1.9 Research Aim

Loquat (*Eriobotrya japonica* Lindl) belongs to the Rosaceae family; it is native to China but it has been commercially cultivated in many countries; being highly appreciated for its light, refreshing taste and health-promoting properties. Therefore, although initially considered as an underutilized crop, nowadays loquat has gained added value as it is available during late winter- early spring period. However, it is considered as a largely unexplored crop, since many pre- and postharvest factors still remain unidentified. The current study was composed of 5 independent, yet interrelated experiments. Initially the carotenoid composition in peel and flesh tissue of 'Obusa' fruits, a yellow-fleshed cultivar, in correlation with the progress of fruit maturity

[3] Flesh browning (A), bruising (B), russeting (C), purple spot (D), and a sound fruit (E). The first row shows the intact loquats, the second row shows the same loquats without peel, and the third row the half-cut loquats.

through an analytical and molecular approach was monitored. Towards this aim, high-resolution temporal expression profiles of carotenoid biosynthetic genes in both tissues were determined and linked with individual carotenoids.

Subsequently, the aim was to shed some light in the mechanism behind the development and regulation of purple spot in loquat fruit with the employment of quantitative real-time RT-PCR. In order to further dissect the effect of the sugar measurements on the PS a molecular approach was applied with the investigation of 15 key genes that are involved in the chlorogenic acid biosynthesis (*PAL1, PAL2, C3H* and *PPO1*) and sucrose biosynthesis (*CWI2, CWI3, SPS, SPS1, SPS2, NI2, NI3, SUSY, HXK, FRK* and *VI*).

Another objective of the current study was to dissect changes on qualitative attributes of the two indigenous loquat cultivars grown in Cyprus in correlation with the harvest period, through the evaluation of mechanical properties, qualitative attributes, postharvest performance and bioactive content, with special reference to both free and bound phenolic compounds.

The application of different pre-harvest treatments on the predominant loquat cultivar cv. ‘Karantoki’ in Cyprus was studied. Subsequently in order to study the effect of the preharvest application of five different treatments in correlation with the harvesting period and the evaluation of their impact on the mechanical properties, qualitative attributes and postharvest performance of the loquat fruit.

Finally, and taking into consideration that relatively few data exist about storage potential and postharvest performance, the investigation of the effect of modified atmosphere packaging (MAP) on fruit from the two indigenous loquat cultivars subjected to cold storage in conventional refrigerator was dissected.

2 Research Methodology

2.1 Fruit material

Trees with contiguous vigor and canopy uniformity of three loquat cultivars (‘Morphitiki’, ‘Karantoki’, ‘Obusa’) grown in a commercial orchard in Cyprus [Episkopi (34° 40’ 15' N, 32° 54′ 7' E), Lemesos] were used for the experimental purposes of the current PhD program. The orchard was covered, with white 17-mesh net for protection against extreme weather conditions (hail, strong winds, sunshine) and bird control. The fruit material and experimental design per study are analytically described in the ‘Materials and methods’ sections of §3-7.

2.2 Quality attributes

2.2.1 Weight loss (%)

Weight loss (WL) % was calculated as follows: (A-B) / A × 100, where A was the fruit weight at harvest and B was the fruit weight at shelf life period according to the experiment considered.

2.2.2 Flesh firmness

Flesh firmness (FF), was measured at the two diametrical points on the equatorial region with a texture analyzer (TA.XT plus, Stable Micro Systems, Surrey, U.K.), using a 3 mm diameter probe at a speed of 1 mm/s with a penetration depth of 5 mm and results were expressed in Newtons (**Figure 2.1)**.

2.2.3 Color parameters

Color parameters of fruit peel were determined using the CR-400, Konica Minolta reflection colorimeter (**Figure 2.2**) to indicate the coordinates L* (brightness or lightness; 0= black, 100= white), a* (-a*= greenness, +a*= redness) and b* (-b*= blueness, +b*= yellowness). Two measurements were made diametrically from equatorial sites on each fruit. Hue angle [(H°) (0° = red-purple, 90° = yellow, 180° = bluish-green, 270° = blue)] and Chroma (degree of departure from grey to pure

chromatic color) were also calculated as $\tan^{-1}$ (b*/a*) and $(a^{*2} + b^{*2})^{½}$ respectively. The ratio a*/b* was also calculated.

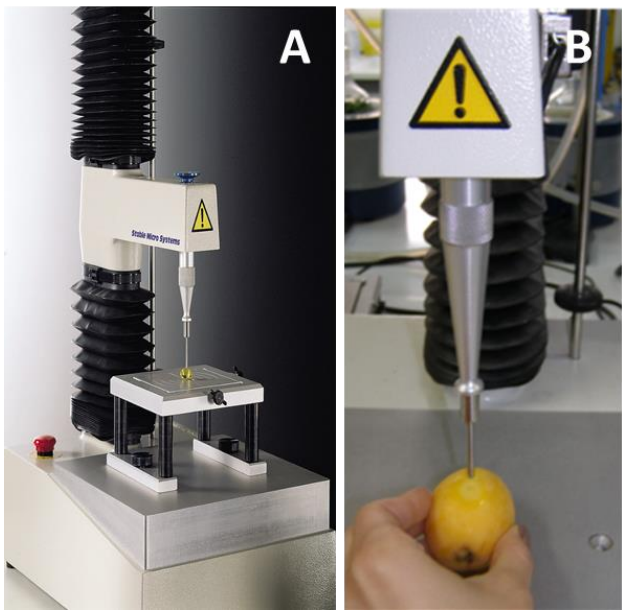

Figure 2.1: Texture analyzer employed with a 3mm diameter probe (A) and the procedure of flesh firmness determination on loquat fruit (B).

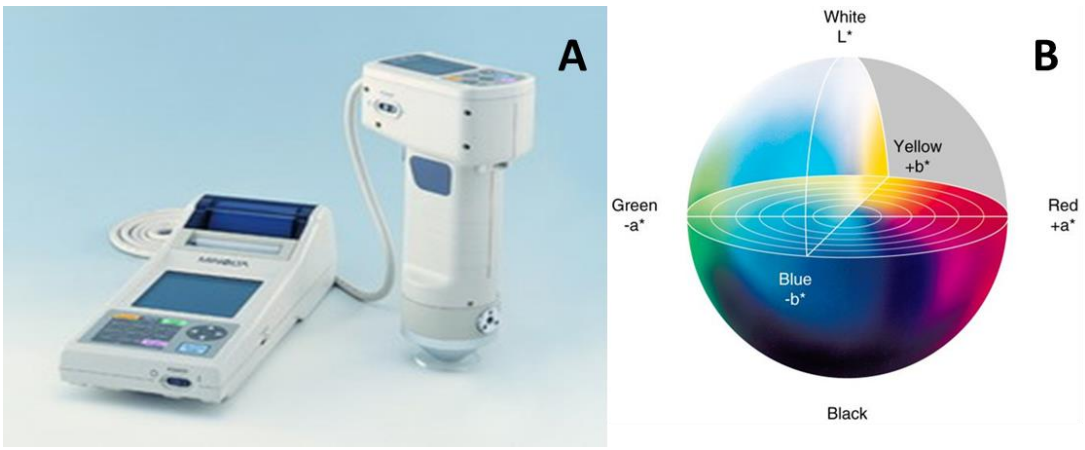

Figure 2.2: CR-400, Konica Minolta reflection chromatometer (A) and L*, a*, b* values according to the Hunter scale (B).

2.2.4 Soluble solids content

A professional juicer was used in order to extract the juice out of 10 fruits (three replicates for each treatment). For the quantification of the soluble solids content (SSC), a PAL refractometer (Atago, PR-32$^{\alpha}$) was employed (**Figure 2.3**). After normalizing the refractometer with distilled water, one drop of fruit juice was placed onto the special lens and measurements were expressed in °Brix.

Figure 2.3: PAL refractometer for determination of SSC.

2.2.5 Individual sugars determination

Peel tissue was used to determine total soluble sugars (TSS), sucrose, glucose and fructose for the needs of the experiment dealing with the purple spot incidence (§4). The sample (0.16 g) was extracted with 5 mL 80% (v/v) ethanol and vortexed. The extracts were then incubated at 85^{o}C for 5 min and subsequently were centrifuged (21191 x *g* for 10 min at 4 ºC). The supernatant was removed and the pellet was re-extracted three additional times in 80% (v/v) ethanol as above, until the extract was colorless. Supernatants from each extraction were pooled, dried at 45 ^{o}C under vacuum, re-solubilized in 15 ml of hot distilled water, and vortexed. After 45 min incubation in an ultrasonic bath (Ultrasonic Cleaner, Raypa, UCI-150) at 40 °C, crude extract supernatants were decanted and held at -20 °C until further analysis.

Total soluble sugars (TSS) were analysed by reacting 0.1 mL of the alcoholic extract with 3 mL fleshly prepared anthrone (150 mg anthrone and 100 mL 72% v/v

H_2SO_4) in boiling water bath (40 °C) for 10 min and then cooled. TSS was then calculated spectrophotometrically at 625 nm (TECAN, Infinite 200® PRO) using sucrose as a standard (Jin et al., 2007).

Sucrose contents were determined by first degrading the reactive sugars present in 0.1 mL extract with 0.1 mL 5.4 M KOH at 95 °C for 10 min. Following the cooling of the reactive product, 3 mL of freshly prepared anthrone reagent were added and the mixture was again heated at 95 °C for 5 min, cooled, and measurements were taken at 620 nm using sucrose as a standard (Jin et al., 2007).

For glucose, 1 mL of the alcoholic extract was heated with 5 mL *o*-toluidine reagent (15 mL *o*-toluidine and 0.5 g thiourea reaching a volume of 250 mL with the use of glacial acetic acid) for 15 min at 95 °C. Absorbance was measured at 630 nm using glucose as a standard (Jin et al., 2007). For fructose, 2 mL of the extract was added to 1 mL of resorcinol reagent (0.6 g resorcinol and 0.15 g thiourea reaching 100 mL glacial acetic acid) and 7 mL HCL/water (5/1, v/v). From the working standard, six different volumes (0, 0.2, 0.4, 0.6, 0.8 and 1 mL) were placed into different tubes and reached the volume of 2 mL with distilled water. Thereafter 1 mL of resorcinol reagent and 7 mL of HCL were added and all tubes were heated in water bath at 80 °C for 10 min. After the mixture was cooled, measurements were made at 520 nm using fructose as a standard (Edewor-Kuponiyi, 2013).

2.2.6 Titratable Acidity

Titratable acidity (TA) was determined with the use of an Automatic multiple positions titrator [862 Compact Titrosampler, Metrohm AG, Switzerland, (**Figure 2.4**). For each measurement, 5 mL of juice was used for titrating 0.1 N NaOH to a pH end point of 8.1. Results were expressed as g malic acid per 100 g fresh weight (FW). With the multiple positions titrator, ten samples were prepared and measured simultaneously.

Figure 2.4: Automatic multiple positions titrator (862 Compact Titrosampler, Metrohm AG, Switzerland) for the TA determination expressed as malic acid equivalents.

2.3 Severity index of purple spot

Harvested fruits per cultivar were segragated into five distinct groups according to the severity incidence (SI) of the purple spot appearance. The group with asymptomatic fruit was coded as NFSI-1, while those showing purple spot symptoms were named as follow: fruit with severity incidence 10-30% was assigned to group NFSI-2, 30-50% affected fruit to NFSI-3 group, 50-70% as NFSI-4 and fruit with 70% and over of total surface was included separated into NFSI-5.

Severity Index (SI) was calculated as follows:

SI = [(NFSI-5 x 1)+(NFSI-4x0.75)+(NFSI-3x0.5)+(NFSI-2x0.25)+(NFSI-1x0)] / tNF,

where NF: number of fruit, tNF: total number of fruit.

2.4 Carotenoid and chlorophyll content determination

Twenty mL of acetone–hexane (4:6, v/v) were added to 100 mg of lyophilised plant material and thoroughly mixed for 2 min. When the two phases were separated, an aliquot was taken from the supernatant and the spectrophotometric absorbance at 663, 645, 505, and 453 nm was determined (**Figure 2.5; Figure 2.6**).

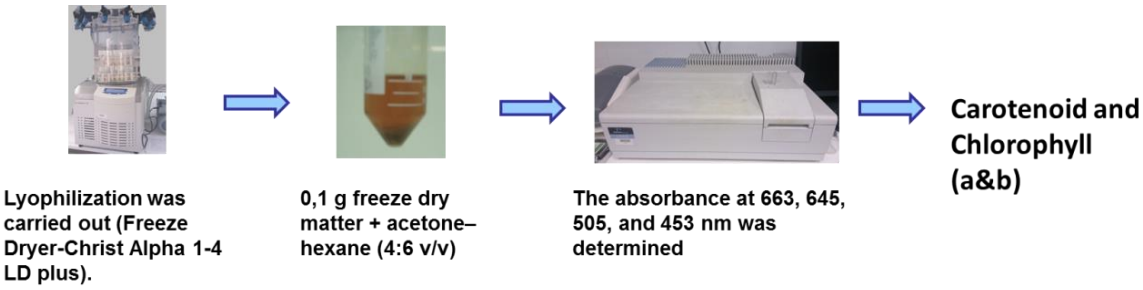

Figure 2.5: Carotenoid and chlorophyll content spectrophotometric determination.

Chlorophyll-a, chlorophyll-b, and b-carotene contents were calculated according to the following Nagata and Yamashita equations (1992):

Chlorophyll a (mg/100ml) = 0.999 A663 - 0.0989 A645
Chlorophyll b (mg/100ml) = -0.328 A663 + 1.77 A645

β-Carotene (mg/100ml) = 0.216 A663 - 1.22 A645 - 0.304 A505 + 0.452

The values A663, A645, A505 and A453 represent absorbance at 663 nm, 645 nm, 505 nm and 453 nm respectively.

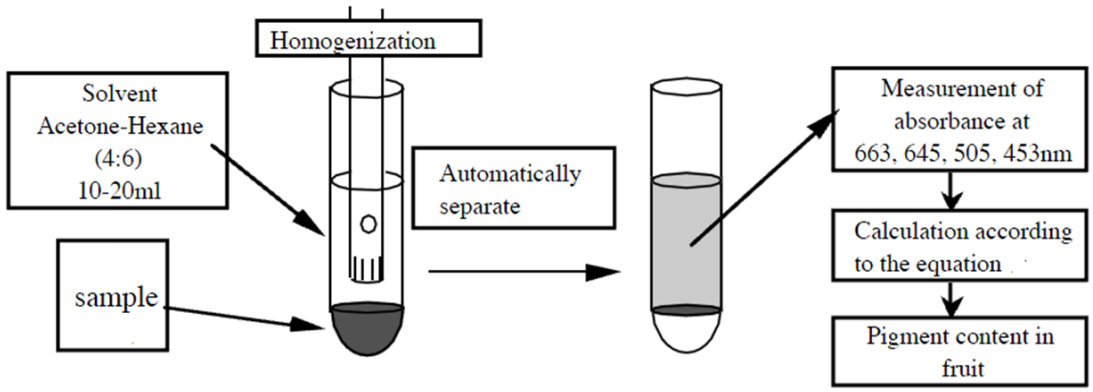

Figure 2.6: Schematic representation of chlorophyll and carotenoid content determination (Source: Nagata and Yamashita, 1992).

2.5 Extraction and determination of free and bound phenolics[4]

Five grams of fine powdered flesh tissue was homogenized with 15 mL of 950 mL L^{-1} cold ethanol, vortexed and centrifuged at 4223 x *g* for 10 min. The pellet was then re-extracted with 10 mL of 800 mL L^{-1} cold ethanol. The supernatants were

[4] Employed in the third experiment (§5), that analysed the impact of harvesting day

combined to make a final volume of 25 mL (Cao et al., 2009). Following the free phenolics extraction, 10 mL of 4 M NaOH were added in the pellet. The suspension was then sonicated for 90 min at 40 °C. Following alkaline hydrolysis, the solution was acidified to pH 2.0 with concentrated HCl. Then, the mixture was centrifuged at 4223 x *g* for 5 min to remove the cloudy precipitates. The liberated phenolic acids (PAs) were then extracted in the clear solution 3 times with 15 mL ethyl acetate and the upper phase was transferred in clean falcon for a total volume of 45 mL. The pooled ethyl acetate extracts were then evaporated to dryness at 45 °C. Subsequently, the dry residue was dissolved in 5 mL of ethanol and sonicated for 15 min (Irakli et al., 2012).

Aliquots of 100 µL of free and bound phenolic extracts were reacted with 500 µL of Folin-Ciocalteu and 4.8 mL of deionized water. The mixture was left to set for 3 min. Then, 1 mL of saturated sodium carbonate and 3.5 mL deionized water were added and left for 1 h in the dark at room temperature. The absorbance was measured at 725 nm (TECAN, Infinite 200® PRO; **Figure 2.7 A**) and results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} FW (**Figure 2.7 B**).

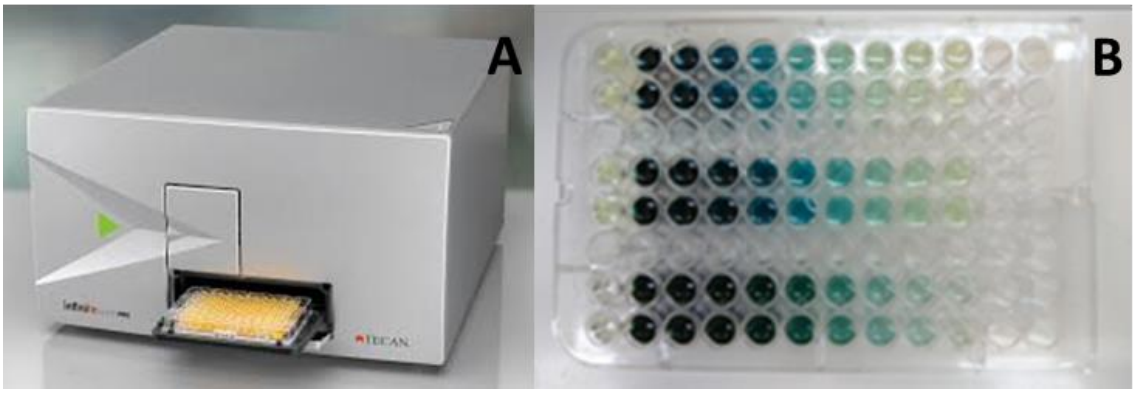

Figure 2.7: TECAN, Infinite 200® PRO used for the absorbance measurements (A) and the chromatic results of the gallic acid standard curve measurements (B).

2.6 Determination of total antioxidant capacity[5]

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity was determined according to Goulas et al. (2014). One of the few stable organic nitrogen radicals is DPPH.The principle of the method is based on the consumption of DPPH radical by

[5] Employed in the third experiment that analysed the impact of harvesting day (§5)

antioxidants, ie the ability of the antioxidants to give phenolic hydroxyl hydrogen to the free radical. This results in the discoloration of the DPPH root solution which changes from bright purple to yellow following the complete consumption of the root by antioxidants. For the DPPH determination 500 μL of the free phenolics sample and 200 μL of bound phenolics were mixed with 2 mL DPPH 0.135 mM. The mixtures were then incubated in the dark for 30 min and the absorbance was measured at 517 nm. A standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared and results were expressed in μmol 100^{-1} FW.

The antioxidant capacity was additionally determined by ferric acid reducing antioxidant power (FRAP) method as elsewhere described (Goulas et al., 2014**)**. The method is based on the conversion of Fe (lll) with the use of TPTZ (Tripyridil-s-triazine) to Fe (ll) as the initial yellow color (Fe (lll) + TPTZ) receives a hydrogen from the antioxidants and is being converted to Fe (ll) (blue color). A sample containing 3 mL FRAP solution and 250 μL of the free phenolics sample or 100 μL of bound phenolics sample was incubated in water-bath (37°C) for 4 min. A standard curve of Trolox was also prepared. The absorbance was measured at 593 nm and results were expressed as μmol Trolox 100 g^{-1} FW.

2.7 Liquid Chromatography Mass Spectrometry (LC-MS) analysis of carotenoids[6]

2.7.1 Extraction and saponification of loquat carotenoids

Liquid-liquid extraction and saponification of the samples was carried out as previously described by Minguez-Mosquera and Hornero-Mendez (1993). Briefly, 0.5 g of freeze-dried tissue (peel or flesh) was homogenized in acetone-butylated hydroxytoluene - 0.1% using an UltraTurrax (Ika, Staufen, Germany) and centrifuged at 2000 r.p.m for 10 min at 4 ˚C. Extraction steps were repeated until complete removal of colour in the sample. The internal standard used was β-Apo-8′-carotenal (Sigma, St Louis, MO, USA). The extracts were combined and treated with diethyl ether. A solution of NaCl (10%, w/v) was added to separate the phases.

[6] Employed in the first experiment that dealt with carotenoid identification (§3)

The lower phase was discarded and the remaining phase was washed with Na2SO4 (2%, w/v) to remove water residues. Fifty mL of a methanolic solution of KOH (20%, w/v) was added and left for 1h in darkness. The organic phase was washed several times with deionized water until washings were neutral. It was then filtered through a bed of anhydrous Na2SO4 and evaporated until dry using a speed vacuum (Thermo Scientific Savant SPD121P). The pigments were collected with 1mL of acetone: methanol (7:3, v/v) and stored at -20 ˚C until analysis. All operations were carried out under pale light to prevent isomerization and photodegradation of carotenoids.

2.7.2 LC- MS analysis of loquat carotenoids

The carotenoid analysis was carried out using an Agilent 1200 HPLC equipped with a photodiode array detector and a single quadrupole mass spectrometer detector in series (6120 Quadrupole, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a reverse-phase Poroshell 120 EC-C18 column (100 mm × 3 mm, 2.7 μm particle size) (Agilent Technologies) operating at 32 ºC. Water with 0.05 M ammonium acetate and acetonitrile: methanol (70:30) were used as mobile phases A and B, respectively, with a flow rate of 0.7 mL min-1. The linear gradient started with 60% of solvent B in A, reaching 100% solvent B at 20 min; this was maintained up to 35 min. The initial conditions were re-established at 36 min and kept under isocratic conditions up to 40 min. Injection volume was 5 μL. All of the carotenoids and carotenoid esters were detected and quantified in UV-vis at 450 nm (**Supplementary Figure S1**).

The identification of carotenoids in loquat was carried out on an Agilent 1100 HPLC system equipped with a photodiode array and an ion trap mass spectrometer detector (Agilent Technologies, Waldbronn, Germany). The mass detector was a Bruker ion trap spectrometer (model HCT Ultra) equipped with an APCI (Atmospheric Pressure Chemical Ionization). The mass spectrometer parameters were as follows: positive ion mode (APCI +); source temperature, 350 ºC; probe temperature, 450 ºC, corona voltage, 4.0 kV; The full scan mass covered the range from m/z 100 up to m/z 1200 and the target mass was adjusted to 350. Collision-induced fragmentation

experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V.

Mass spectrometry data were acquired in the positive mode, and the MSn was carried out in the automatic mode. The identification of the peaks was performed by the extracted ion-chromatograms of the ion current at m/z values corresponding to the $[M-H]^+$ ions of the individually investigated compounds, as well as their fragmentation. Furthermore, to confirm the identification of the carotenoids and obtain a more reliable identification, samples were analyzed with the Agilent 1290 Infinity UPLC system coupled to a quadrupole (Q-TOF) mass spectrometer (6550 Accurate-Mass QTOF, Agilent Technologies) using an electrospray interface with jet stream technology.

The chromatographic separation was developed under the same conditions, as described above. The optimal conditions for the electrospray interface were as follows: gas temperature: 300 ºC, drying gas 11L/min, nebulizer 65 psi, sheath gas temperature 400 ºC, sheath gas flow 12 L/min. Spectra were acquired in the m/z range of 100-1100, in a positive mode and with an acquisition rate of 1.5 spectra in MS, maintaining a mass resolution over 50,000 for the mass range used. Internal mass calibration by the simultaneous acquisition of reference ions and mass drift compensation was used for obtaining low mass errors. Q-TOF MS data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00). For quantification, β-apo-8'-carotenal was used as an internal standard. Lutein, β-carotene and violaxanthin (Sigma, St Louis, MO, USA) in a concentration range of 5-100 $\mu g.mL^{-1}$ were used to quantify compounds in three different groups, hydroxycarotenoids, carotenes, and epoxycarotenoids, respectively. Neoxanthin, neochrome, β-cryptoxanthin epoxides and β-carotene epoxides were estimated as violaxanthin. β-Cryptoxanthin was quantitated as lutein. The *cis*-isomers were quantitated with the calibration curve of the all-trans isomers. Concentrations were micrograms of pigment per 100 g of sample fresh weight ($\mu g\ 100\ g^{-1}$ FW).

2.8 RNA extraction [7]

Total RNA was extracted from three bulked biological replicates of loquat fruit material for each developmental stage (S1-S6) according to a modified cetyltrimethylammonium bromide (CTAB) protocol developed by Gambino et al. (2008). Next, RNA integrity was confirmed spectrophotometrically (Nanodrop 1000 Spectrophotometer, Thermo Scientific) followed by gel electrophoresis and samples were treated with RNase-free DNase (Cat. No. NU01a, HT Biotechnology LTD, England) to remove total gDNA (**Figure 2.8**) according to the procedure described in Georgiadou et al. (2016).

For this procedure 0.5 mL (1unit) of RNase-free DNase was added to the extracts and ddH2O was added to a final volume of 50 mL. Samples were mixed and incubated at 37°C for 30 min. The enzyme was heat-inactivated at 75°C (5min) and the volume was raised to 150 mL with the addition of ddH_2O. Subsequently, 1/10 volume of 3M CH3COONa (pH D 4.8) and 2.5 volumes of absolute ethanol were added, and the samples were briefly vortexed and incubated at 80°C overnight. RNA was precipitated by centrifugation at 16000 g for 30 min at 4 °C (Eppendorf Centrifuge 5415 R, Germany).The supernatant was discarded and the Eppendorf tubes were dried at 50°C for 2 - 3 min. RNA was dissolved in 20 mL ddH2O. The RNA integrity was examined spectrophotometrically (Nanodrop 1000 Spectrophotometer, Thermo Scientific, USA) and by gel electrophoresis before storing at 20 °C.

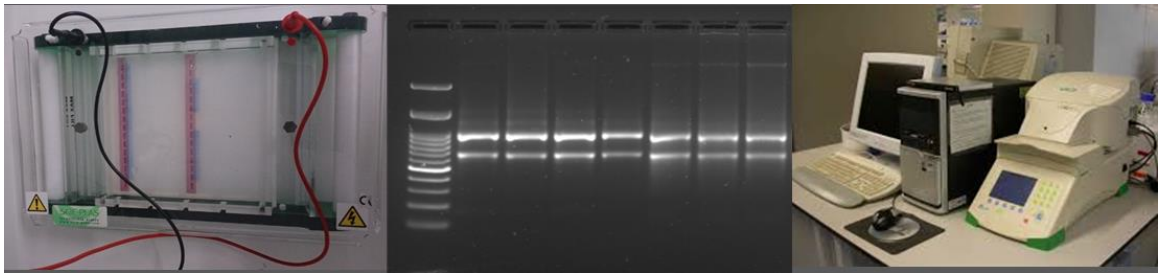

Figure 2.8: Gel electrophoresis procedure followed by the removal of the total gDNA and total RNA quantification with real-time RT-PCR.

[7] Employed in the first experiment (§3), that dealt with carotenoid identification and the second experiment (§4) that dealt with the purple spot incidence

For the second experiment ‘Obusa’ and ‘Karantoki’ fruits with purple spot severity of 30-50 % were selected for gene expression analysis. Total RNA was extracted from peel tissue (both asymptomatic and symptomatic tissue from the same fruit) of three fruits (biological replicates) per cultivar. In the case of ‘Morphitiki’, only healthy peel tissue samples were excised, and samples were prepared as described above.

2.9 cDNA synthesis and Quantitative real-time RT-PCR analysis

For real-time RT-PCR analyses, 0.5 μg of total RNA was reverse transcribed using the PrimeScriptTM RT reagent Kit (Takara Bio, Japan), according to the manufacturer's protocol (Takara Bio, Japan). Subsequently, quantitative real-time RT-PCR was performed with BioRad IQ5 real-time PCR cycler (BioRad, USA). In total, three biological replicates were performed for each developmental stage for both loquat peel and flesh.

For the first experiment, that dealt with carotenoid identification the reaction mixture consisted of 4 μL cDNA in reaction buffer (15-fold diluted first-strand cDNA for all genes except *VDE* and *LCYE* that was 5-fold diluted), 0.5 μL of each primer (10 pmol/μL) and 5 μL SensiFASTTM SYBR® & Fluorescein mix 2x (Bioline). The total reaction volume was 10 μL. The initial denaturation step was at 95^{o}C for 5min, followed by 40 cycles of amplification [95 ^{o}C for 30 s, annealing temperature (Ta ^{o}C) for 30 s, and 72 ^{o}C for 30 s] and a final elongation stage at 72 ^{o}C for 5 min. Gene amplification cycle was followed by a melting curve run, carrying out 61 cycles with 0.5 ^{o}C increment from 65 to 95 ^{o}C. The annealing temperature (58 to 65 ^{o}C) of previously published primers for loquat carotenoid biosynthetic genes (**Supplementary Table S1)**. Loquat’s Actin gene was used as a housekeeping reference gene (*EjACT*).

For the second experiment that dealt with the purple spot incidence, three biological replicates were performed for cvs. ‘Karantoki’ and ‘Obusa’, on both symptomatic (30-50%) and asymptomatic peel tissue at fully-ripe stage. For cv. ‘Morphitiki’ only asymptomatic peel tissue at fully-ripe stage was excised. The reaction mixture of 10 μL consisted of 4 μL cDNA in reaction buffer (10-fold diluted first-strand cDNA for all genes except for *PAL2* that was diluted 5-fold), 0.5 μL of each primer (10

pmol/μL) and 5 μL SensiFAST™ SYBR® and Fluorescein mix 2x (Bioline). Thermal profile initiate with a denaturation step was at 95°C for 5 min, followed by 40 cycles of amplification [95 °C for 30 s, annealing temperature (Ta °C) for 30 s, and 72 °C for 30 s] and a concluding elongation stage at 72 °C for 5 min. This gene amplification cycle was followed by a melting curve run, of 61 cycles with 0.5 °C increment from 65 to 95 °C. The primer information related to polyphenolic and carbohydrate biosynthetic genes (50-65°C) are listed in **Supplementary Table S2.** Loquat's *actin* gene was used as a housekeeping reference gene (*ACT).*

2.10 Statistical analysis

The statistical analyses per experiment are analytically described in the 'Statistical analysis' section of each experiment (chapters §3-7).

3 Metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway on peel and flesh tissue of loquat fruit during on-tree development

3.1 Abstract

Carotenoids are the main colouring substances found in orange-fleshed loquat fruits. The aim of the current study was to unravel the carotenoid biosynthetic pathway of loquat fruit (cv. 'Obusa') in distinct tissues (peel and flesh) during distinct on-tree development through a targeted analytical and molecular approach. Substantial changes regarding colour parameters, both between peel and flesh and among the different developmental stages, were monitored, concomitant with a significant increment in carotenoid content. Key genes and individual compounds that are implicated in the carotenoid biosynthetic pathway were further dissected with the employment of molecular (RT-qPCR) and advanced analytical techniques (LC-MS). Results revealed great differences in carotenoid composition between peel and flesh. Thirty-two carotenoids were found in the peel, while only eighteen carotenoids were identified in the flesh. The major carotenoids in peel were *trans*-lutein and *trans*-β-carotene; the content of the former decreased with the progress of ripening, while the latter registered a 7.2-fold increase. However, carotenoid profiling of loquat flesh revealed *trans*-β-cryptoxanthin, followed by *trans*-β-carotene and 8-epoxy-β-carotene to be the most predominant carotenoids. High amounts of *trans*-β-carotene in both tissues was supported by significant induction in *a* chromoplast-specific *lycopene β-cyclase (CYCB)* gene expression. *PSY1*, *ZDS*, *CYCB*, and *BCH* were upregulated and *CRTISO*, *LCYE*, *ECH* and *VDE* were down-regulated in most of the developmental stages compared to the immature stage in both peel and flesh tissue. Overall, more pronounced levels of differential regulation of gene expression with the progress of on-tree fruit development was monitored in the middle and downstream genes of carotenoid biosynthetic pathway. Carotenoid composition is greatly affected during on-tree loquat development with striking differences between peel and flesh tissue. A link between gene up- or down-regulation during the developmental stages of the loquat fruit, and how their expression affects carotenoid content per tissue (peel or flesh) was established.

Keywords: *Eriobotrya japonica*, developmental stages, maturation, ripening, β-carotene; β-cryptoxanthin, lutein, LC-MS, biosynthetic pathway

3.2 Introduction

Carotenoids play an important role in loquat, as they affect organoleptic characteristics and health effects of the fruit. In particular, carotenoids are the main pigments in loquat and impact flavor acceptability, since they are precursors of important volatile flavor compounds (Vogel et al., 2010). Regarding nutraceutical properties of fruit carotenoids, numerous studies have revealed several physiological responses to carotenoids that may be relevant to the promotion of health and the prevention or treatment of some chronic diseases (Kaulmann et al., 2014). In particular, fruits containing carotenoids have been linked to the prevention of cancer, inflammation, cardiovascular diseases, and cataract (Zhou et al., 2007; De Faria et al., 2009; Fu et al., 2012) and are also known to enhance immune responses (Zhou et al., 2007; De Faria et al., 2009; Kato, 2012). Carotenoid profile in loquat is influenced by maturity stage, environmental and most promptly genetic factors. Loquat cultivars have been segregated to white- and red-fleshed (Fu et al., 2012; Fu et al., 2014). However, segregation of loquat cultivars based on their flesh color can be confusing, since additionally the terms yellow- and orange-fleshed are being used. White-fleshed cultivars have a creamy, pale yellow color, while the terms red- and orange-fleshed can be considered as synonymous. The latter type cultivars have higher carotenoid concentrations than the lighter coloured ones (Zhou et al., 2007; De Faria et al., 2009; Fu et al., 2012; Fu et al., 2014; Goulas et al., 2014).

Carotenoids are formed from isopentenyl diphosphate (IPP), a five-carbon compound, and dimethylallyl diphosphate (DMAPP), its allylic isomer. These form geranylgeranyl diphosphate (GGPP) which in turn forms phytoene through the activity of *phytoene synthase (PSY)*. Phytoene forms lycopene via four desaturation reactions with the involvement of *phytoene desaturase (PDS) ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS)* and *carotene isomerase (CRTISO)* (Naik et al., 2003). Lycopene, in turn undergoes a series of reactions to form lutein, through the ε, β-branch, the predominant carotenoid pigment in green plants (Naik et al., 2003), and violaxanthin

from zeaxanthin with the presence of *zeaxanthin epoxidase (ZEP)* though the β, β-branch (**Figure 3.1)**.

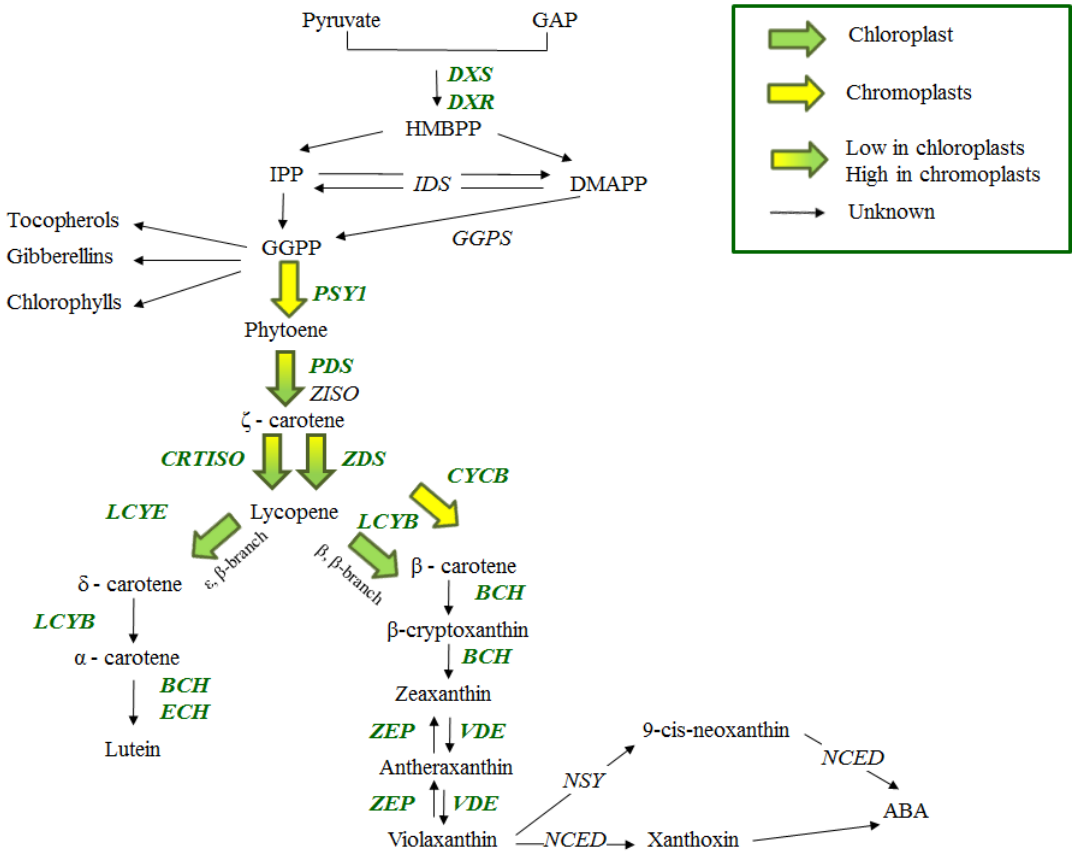

Figure 3.1: Carotenoid biosynthetic pathway in loquat fruit. Genes examined are in green italic bold letters. (Figure is modified from Naik et al., 2003; Galpaz et al., 2006; Zhao et al., 2011; Fu et al., 2012; Kato, 2012).[8]

This forms the xanthophyll cycle, the mechanism that enables plant adaptation to high light stress (Fu et al., 2014). 9-cis-neoxanthin is derived from the conversion of violaxanthin by *neoxanthin synthase (NSY)*, which in turn forms the phytohormone

[8] *The enzymes/genes are: DXS, 1-deoxy-D-xylulose 5-phosphate-synthase; DXR ,DXP reductoisomerase; IDS, isopentenyl pyrophosphate synthase; IDI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl diphosphate synthase; PSY1, phytoene synthase; PDS, phytoene desaturase; ZISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene β-cyclase; CYCB, chromoplast-specific lycopene β-cyclase; LCYE, lycopene ε-cyclase; BCH, β-carotene hydroxylase; ECH, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED 9-cis-epoxycarotenoid dioxygenase. The metabolites are: pyruvate; GAP, D-glyceraldehyde 3-phosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GGPP, geranylgeranyl diphosphate; Phytoene; ζ – carotene; lycopene; α – carotene; β – carotene; δ - carotene; lutein; β-cryptoxanthin; zeaxanthin; antheraxanthin; violaxanthin; neoxanthin; xanthoxin; ABA, abscisic acid.*

abscisic acid through *9-cic-epoxycarotenoid dioxygenase (NCED)* activity (Kato, 2012; Mendes et al., 2015), which controls abiotic stress signalling pathways.

The carotenoid biosynthetic pathway is controlled by the presence of the key enzyme PSY (**Figure 3.1**, Naik et al., 2003; Galpaz et al., 2006; Zhao et al., 2011; Fu et al., 2012; Kato, 2012; Shumskaya and Wurtzel, 2013). Regarding loquat fruit, Fu et al. (2012) investigated the mechanism underlying the differentiation of carotenoids in a red-fleshed (cv. 'Luoyangqing') and a white-fleshed (cv. 'Baisha') cultivar; differences in carotenoid accumulation in the two cultivars were linked with the differential expression of *PSYI, CYCB,* and *BCH* genes.

The aim of the current study was to monitor the carotenoid composition in peel and flesh tissue of 'Obusa' fruits, a yellow-fleshed cultivar, in correlation with the progress of fruit maturity. Towards this aim, high-resolution temporal expression profiles of carotenoid biosynthetic genes in both tissues were determined by RT-qPCR and linked with individual carotenoids, quantified by LC-MS.

3.3 Materials and Methods

3.3.1 Fruit material

Loquat fruits cv. 'Obusa' were harvested at ca. 10-day intervals, between March 30th and May 14th. For each developmental stage, 30 fruits (three 10-fruit sub-lots per stage) were harvested based on size uniformity and external colour. The fruit were initially used for the determination of physical dimensions (**Figure 3.2**) and colour and subsequently for molecular and analytical analysis, as described below. The developmental stages were defined using the BBCH scale (Martinez-Calvo et al., 1999). For the molecular analysis fresh samples of both peel and flesh were immediately frozen in liquid nitrogen, freeze-dried and kept at -80°C until further use. For the determination of carotenoid profiles, samples were freeze-dried (Freeze Dryer-Christ Alpha 1-4 LD plus).

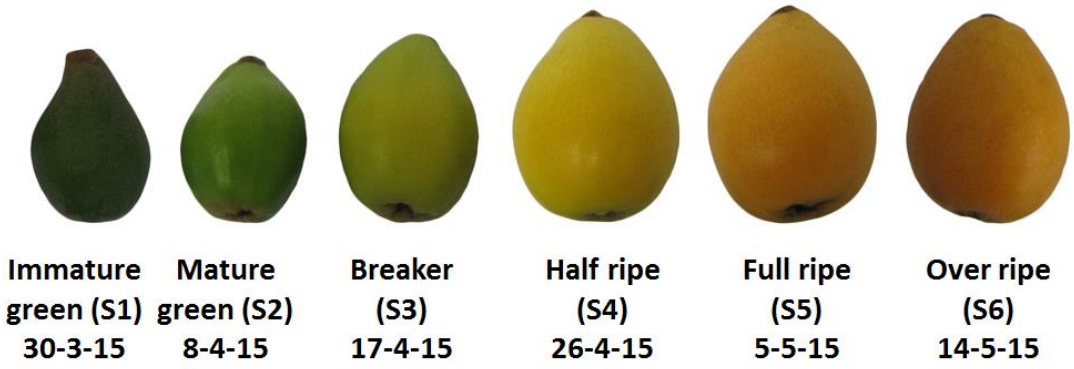

Figure 3.2: Phenotypes of cv 'Obusa' loquat fruit developmental stages; immature green to over ripe (S1-S6).

3.3.2 Methodology

Upon fruit set and immediately after fruit thinning, length (L) and width (W) from ten fruits per cultivar were measured, at 7-day intervals, for twelve weeks with the employment of an electronic caliper (IS11112, Insize).

The colour parameters CIE L*, a* and b* were also measured as described in §2.2.3. The carotenoid and chlorophyll content was measured as described in §2.3. Liquid-liquid extraction and saponification of the samples was carried out as previously described in §2.7.1, followed by the carotenoid analysis §2.7.2. Total RNA was extracted from three bulked biological replicates of loquat fruit material for each developmental stage (S1-S6) as previously described in §2.8 and samples were treated with RNase-free DNase to remove total gDNA as previously described in §2.8. Real-time RT-PCR analyses was carried out according to the protocol described in §2.9.

3.3.3 Statistical analysis

Statistical analyses were carried out by comparing the averages of each developmental stage based on the analysis of variance (one way Anova) according to Duncan's multiple way test with a significance level of 5 % ($P \leq 0,05$), using the statistical analysis software package SPSS v.17.0 (SPSS Inco Chicago, USA). The relative quantification and statistical analysis of gene expression levels using the pairwise fixed reallocation randomization test were performed with the REST-XL software according to Pfaffl et al. (2002). Gene expression levels were normalized

against the *EjACT* housekeeping reference gene; the first developmental stages (S1) both for peel and flesh tissue were used for calibration.

3.4 Results and Discussion

3.4.1 Qualitative attributes

Fruit weight, length and width ranged between 25.3-59.1 g, 48.8-57.0 mm and 34.4-45.0 mm, respectively (**Supplementary Table S3**). Maximum fruit size and weight was recorded at stage S5, which coincides with the optimum maturity stage for harvest.

Colour parameters in the flesh ranged between 52.1-74.3 for L*, -11.5-10.7 for a* and 30.8-36.7 for b*. The corresponding values for L^*,a^*,b^* parameters in the peel were 45.7-65.0, -17.7- 12.4 and 28.2-49.2 (**Supplementary Table S4**). Previous studies in white and red-fleshed loquat cultivars indicated that the a*/b* chroma ratio was negative for green fruits, zero for yellow fruits, and positive for orange fruits (Zhou et al., 2007). The same study also suggested that a cultivar deeper in red colour was associated with a higher positive a*/b* value. Therefore, the peel colour of the red-fleshed cultivars is also deeper red because of the higher levels of carotenoid accumulation (Zhou et al., 2007). In our study, the a*/b* ratio received negative values at stages S1-S3, implying the green colour, the S4 value indicates the colour break and the S5 and S6 had the higher values. Similarly, the a*/b* ratio was lower in the peel during the first three early stages (-0.6 to -0.2), close to zero in the breaker stage, while it received positive values in the last maturation stages (0.2-0.3) (**Supplementary Table S4**). Overall, the a*/b* ratio can be linked with total carotenoid accumulation and its transient increase with the progress of on-tree development in accordance with previous studies (Zhou et al., 2007; Zhao et al., 2011).

3.4.2 Carotenoid and chlorophyll contents

Initially, a rapid colorimetric assay was employed to screen carotenoid and chlorophyll a and b (Chl a and Chl b) contents. β-carotene varied between 0.8 - 6.1 and

0.1 - 5.9 mg 100 g^{-1} FW β-carotene equivalents in the peel and in the flesh, respectively (**Figure 3.3**). Notably similar values were monitored at S6 stage.

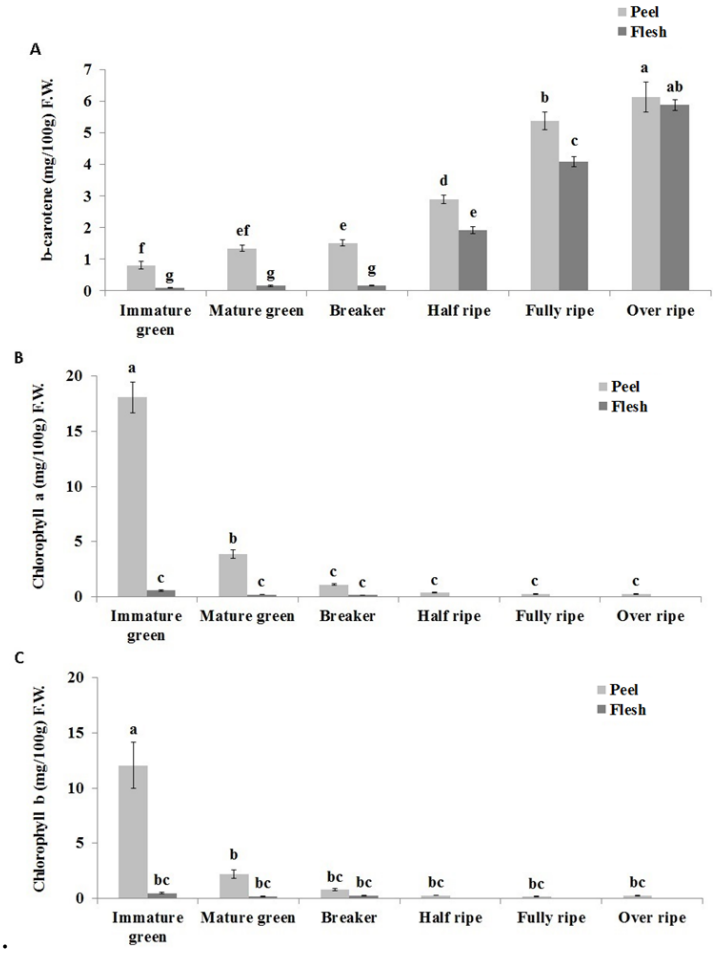

Figure 3.3: A) β-carotene, B) chlorophyll-a, and C) chlorophyll-b content for the 6 developmental stages.[9]

The highest accumulation of carotenoids was observed at the last developmental stage for both peel and flesh. The highest concentrations in Chl a and Chl b were found in the peel tissues at the immature green stage (18.0 and 12.1 mg 100 g^{-1} FW, respectively), while their contents were substantially lower in the flesh (0.6 and 0.5 mg 100 g^{-1} FW at S1 stage, respectively) and degraded thereafter with the progress of on-tree ripening. In particular, during ripening of fleshy fruits, chloroplasts turn into chromoplasts in a process that involves degradation of chlorophylls and a massive

[9] *The statistical analysis for each parameter (β-carotene, Chl a and Chl b) show the statistical difference between the 6 developmental stages. Results expressed as the means ± standard error*

accumulation of carotenoids, causing the change in fruit colour (Lois et al., 2000). Chl a is a blue-green coloured pigment and is less stable than the yellow-green Chl b.

3.4.3 Identification of carotenoids in loquat peel and flesh

Carotenoids in peel and flesh were identified and quantified. Thirty-two carotenoids were detected by HPLC-DAD and LC-MS techniques. Peak identification was based on their relative retention time values, their UV-Vis spectra, their mass spectra, information from the literature and comparison with authentic standards when possible. Supplementary Table S4 summarizes the identification data for each carotenoid, including chromatographic and spectroscopic values. All *trans*-lutein was identified by comparison with an authentic standard. All *trans*-lutein (peak 10) and *cis*-lutein (peak 19) showed characteristic UV-visible spectra, with a hypsochromic shift of 8 nm for the *cis* isomer. The identification of both lutein isomers was confirmed by their mass spectra with the protonated molecule at *m/z* 569 and fragments at *m/z* 551 and *m/z* 533 due to the loss of one and two hydroxyl group respectively. The MS/MS showed, in addition, the presence of fragments at *m/z* 477 resulting from the loss of toluene ([M+H-92] $^{+}$) from the polyene chain. In APCI-MS, the fragment with *m/z* 551 presented a higher intensity than the protonated molecular ion (*m/z* 569).

Peaks 17 and 18 were identified as mono-epoxides of β-cryptoxanthin considering their UV-vis and MS characteristics by the comparison with literature data (De Rosso and Mercadante 2007; De Faria et al., 2009). The mass spectra of both epoxides presented the protonated molecule at *m/z* 569 and fragment ion at *m/z* 551 due to the loss of a hydroxyl group. Peak 17 was designated as 5',6'-epoxy- β-cryptoxanthin due to the presence of the mass fragment at *m/z* 205 that is consistent with the location of one epoxide group in the unsubstituted ring whilst peak 18 showed the mass fragment at *m/z* 221, indicating that the epoxide groups were in a ring with a hydroxyl group. β-Apo-8'-carotenal was identified as peak 21 (internal standard).

The mass spectra presented the protonated molecule at *m/z* 417. Ions of *m/z* 399 and 389 were detected corresponding to the loss of water and carbon monoxide respectively. Elimination of toluene from the protonated molecule was observed at *m/z* 325. The use of an internal standard was recommended to estimate the losses during the extraction process. Saponification with potassium hydroxide has been an integral part of

carotenoid analyses. Kimura et al. (1990) showed that β-apo-8'-carotenal was completely transformed to citranaxanthin (peak 22), apparently by aldol condensation with acetone. The conversion percentage from β-apo-8'-carotenal to citranaxanthin was 98% and their sum was considered for quantification.

The identification of citranaxanthin was confirmed on the basis of its protonated molecule at *m/z* 457 (Shlatterer and Breithaupt, 2006) and its characteristic UV-vis spectrum (Mitrowska et al., 2012). Due to the presence of the same chromophore, β-cryptoxanthin (peak 26) and β-carotene have similar UV-visible spectra. As expected, the protonated molecule was detected at *m/z* 553 and the MS/MS revealed the presence of fragment ions at *m/z* 551 and 461 corresponding to the losses of the hydroxyl group and toluene. 5,8-epoxy-β-carotene (peak 30) could not be identified by its UV-visible spectral characteristics. Mass spectra highlighted the protonated molecule at *m/z* 553 and the MS/MS showed the presence of fragment ions at *m/z* 551 and 461 corresponding to the losses of the hydroxyl group and toluene, respectively, and at *m/z* 221 that corresponds to the location of the epoxide group in the 3-hydroxy-β-ring. The mass spectra of beta-carotene, peak 31, showed the protonated molecule at *m/z* 537 and a fragment ion in the MS/MS at *m/z* 444 corresponding to the loss of the toluene from the polyene chain.

3.4.4 Carotenoid composition

Results revealed great differences in carotenoid composition between peel and flesh. In particular, 32 carotenoids were found in loquat peel, while only eighteen carotenoids were identified in the flesh. Except for qualitative differences, the concentration of carotenoids in peel was significantly higher than that of the flesh. Chromatograms also revealed that the major carotenoids in peel were *trans*-lutein and *trans*-β-carotene. The concentration of *trans*-lutein decreased with the progress of ripening from 1651.5 to 688.4µg 100 g^{-1} FW. On the other hand, *trans*-β-carotene content increased drastically from 151.9 to 1096.9 µg 100 g^{-1} FW. The biosynthesis of some carotenoids such as *trans*-β-cryptoxanthin, 5,8-epoxy-β-carotene, β-diepoxy-cryptoxanthin and *cis*-violaxanthin has also been monitored. Conversely, *trans*-neoxanthin and *trans*-neochrome decreased, indicating that they are catabolized in the carotenoid biosynthetic pathway.

The carotenoid profiling of loquat flesh was found to be quite different from the peel. The most abundant carotenoid in mature fruits was *trans*-β-cryptoxanthin, followed by *trans*-β-carotene, compounds 18 and 31, and 8-epoxy-β-carotene (peak 30) (**Suplementary Table S5; Figure 3.4**). An increment in the concentration of all carotenoids during on-tree development except for *trans*-neoxanthin, *trans*-neochrome and *trans*-lutein was found. Overall, a great effect of the developmental stage on the carotenoid composition was revealed.

All-*trans*-neochrome, all-*trans*-violaxanthin, β- diepoxy-cryptoxanthin, *cis*-violaxanthin, all-*trans*-lutein, 5,6-epoxy-β-cryptoxanthin, 5',6'-epoxy-β-cryptoxanthin, *all*-trans-β-cryptoxanthin, phytoene and all-*trans*-β-carotene were previously identified in five loquat cultivars, originating from Brazil [9]. In their findings, they reported *trans*-β-carotene (19-55%), *trans*-β-cryptoxanthin (18-28%), 5,6:5 ,6 -diepoxy-β-cryptoxanthin (9-18%) and 5,6-epoxy-β-cryptoxanthin (7-10%) to be the main carotenoids. In the flesh, it was found that β-carotene and lutein were the major carotenoids with neoxanthin, violaxanthin, luteoxanthin, 9-*cis*-violaxanthin, phytoene, phytofluene and ζ–carotene also present.

The carotenoid quantification in our study proved that the peel had higher carotenoid concentrations than the flesh, except for β- Diepoxy-cryptoxanthin which was found from the 3rd until the 6th developmental stage in the peel, ranging from 15.0 ± 0.8 to 21.2 ± 1.7 μg 100 g^{-1} FW, as well as in flesh during the last maturity stages (S5 and S6) at 17.6 ±1.2 and 21.4 ± 1.8 μg 100 g^{-1} FW, respectively. *Trans*-neoxanthin and *trans*-neochrome appeared throughout all developmental stages in the peel as well as in the first 4 maturation stages in the flesh. On the other hand, *trans*-violaxanthin increased in the peel with the progress of on-tree ripening (15.6 ± 2.5 to 25.2 ± 4.6 μg 100 g^{-1} FW), while it was detected in the flesh during the last stages (S4-S6) with progressive increase (from 12.5 ± 0.2 to 21.5 ± 1.6 μg 100 g^{-1} FW). Similar findings and trend were observed for *cis*-violaxanthin which ranged from 16.2 ± 2.4 to 23.2 ± 3.0 μg 100 g^{-1} FW in the peel, being detectable in the flesh from S4 stage onwards (12.3 ± 0.1 to 19.1 ± 1.5 μg 100 g^{-1} FW). 5',6'-Epoxy-β-cryptoxanthin and *cis*-lutein were detected exclusively in the peel throughout all developmental stages **(Figure 3.4**). Intriguingly, *trans*-lutein in the peel showed the highest content in the initial developmental stages and went descending thereafter, meanwhile it was found in detectable amounts in the flesh only at

stages 1 and 2, still substantially lower compared to the peel. Citranaxanthin and phytoene have also been identified, although they were not quantified

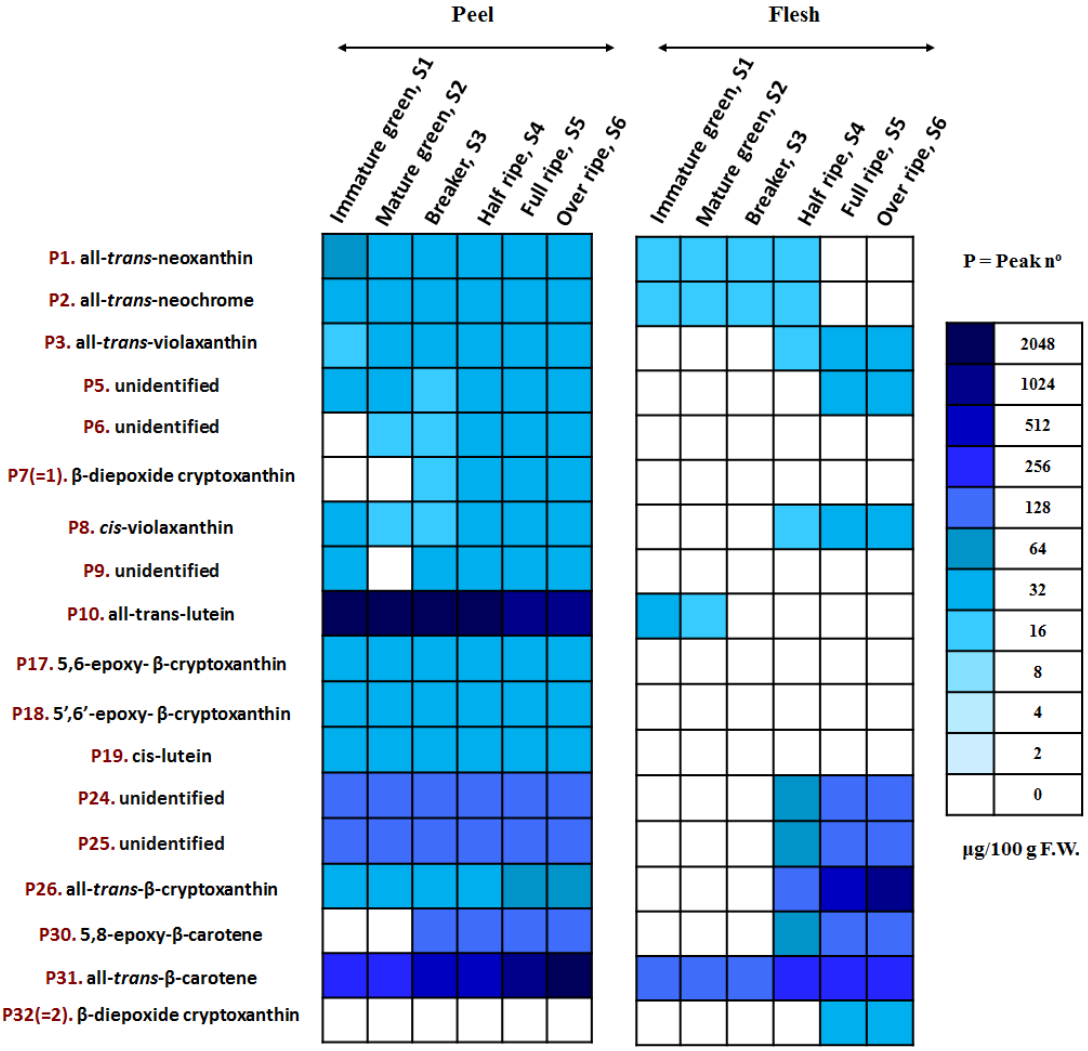

Figure 3.4: Heat map of the quantification of the identified carotenoids both in the peel and in the flesh, of loquat fruit (cv. 'Obusa') during succesive developmental stages.[10]

3.4.5 Gene expression profiling

To elucidate the carotenoid biosynthetic pathway in loquat fruit, the expression profile of thirteen known genes of the carotenoid pathway was analyzed, showing differential expression patterns in peel and flesh tissue **(Figure 3.5**). For gene

[10] *Results are expressed as µg/100 g fresh weight (FW),* (n = 3). *A scale of colour intensity is presented as a legend. Actual quantification of the identified carotenoids are shown in Suplementrary Table S5*

expression analyses each tissue was examined individually, with the calibrator being the S1 stage of the tissue considered (peel or flesh).

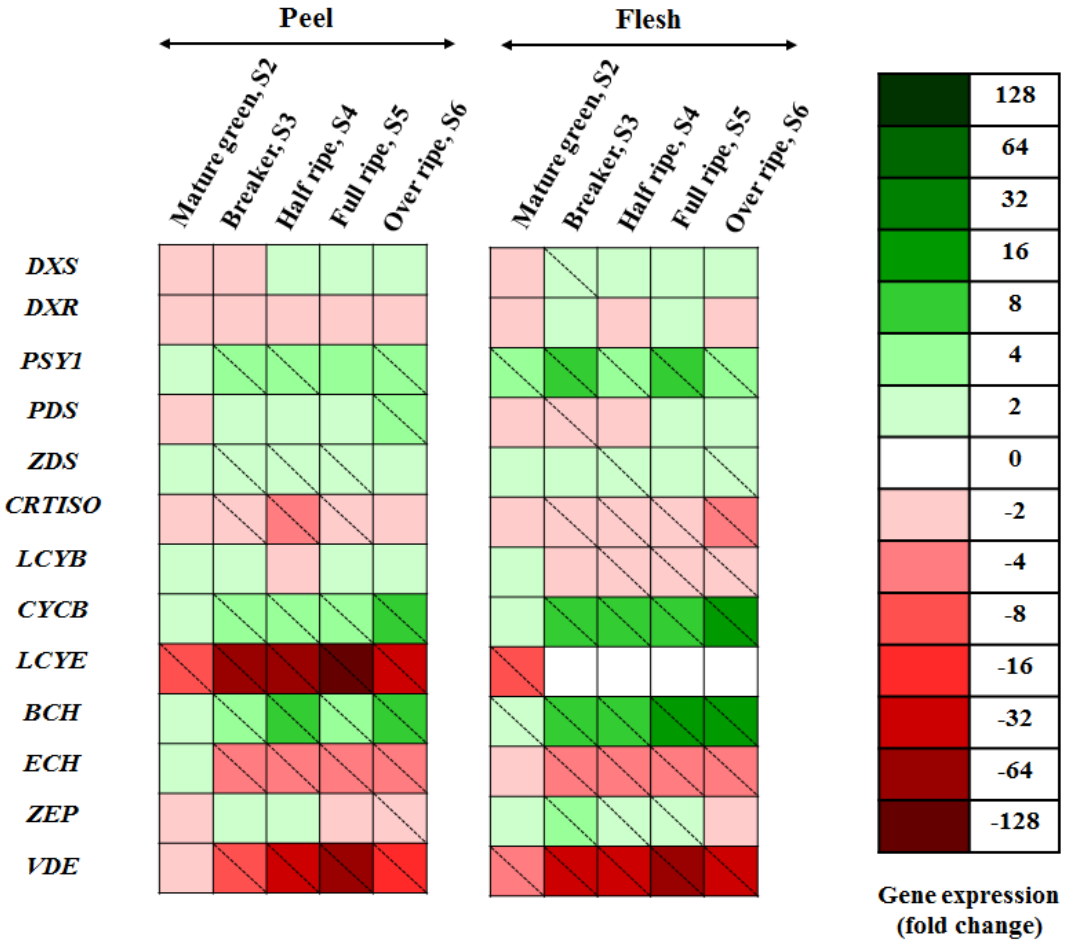

Figure 3.5: Heat map of the relative expression levels of carotenoid biosynthesis genes (*DXS, DXR, PSY1, PDS, ZDS, CRTISO, LCYB, CYCB, LCYE, BCH, ECH, ZEP* and *VDE*) in loquat fruit (cv. 'Obusa'), both in the peel and in the flesh, during 5 developmental stages (S2-S6) (n = 3). Values that differ from the first developmental stage (S1) both for peel and flesh, used as reference.[11]

More pronounced levels of differential regulation of gene expression among S2-S6 developmental stages were monitored in the middle and downstream genes (*CYCB, LCYE, BCH, ECH* and *VDE*) compared with the remaining genes of the carotenoid biosynthetic pathway. In both peel and flesh, *PSY1*, *ZDS*, *CYCB*, and *BCH* were significantly up-regulated in most of the developmental stages compared to the S1 stage

[11] *Relative mRNA abundance was evaluated by real-time RT-PCR using three biological repeats. Up-regulation is indicated in green; down-regulation is indicated in red. A diagonal line in a box indicates a statistically significant value ($P \leq 0.05$). A scale of colour intensity is presented as a legend. Values that differ from the first developmental stage (S1) both for peel and flesh, used as reference.*

respectively; whereas *CRTISO*, *LCYE*, *ECH* and *VDE* were generally down-regulated. *PDS* was significantly up-regulated at the S6 stage in the peel, while it was significantly suppressed in the S3 stage in flesh tissue. *ZDS* presented an increase in expression levels between the S3 and S5 stage in peel, though the increase was at the S4 and S6 stage in flesh tissue. Non statistically significant changes in expression levels of *DXS*, *DXR* (both involved in MEP pathway) and *LCYB* were monitored in the peel in comparison with the S1 stage; in flesh *DXR* followed the same trend whereas, *DXS* presented an accumulation at the S3 and *LCYB* transcript levels decreased gradually through stages S4-S6 which could account for the low or non-detectable *trans*-lutein findings in the flesh (**Figure 3.4; Figure 3.5**). *ZEP* expression levels presented highest suppression at the S6 stage in peel, contrarily to flesh profile where it was up-regulated at the S3-S5 stages.

BCH and *CYCB* transcript levels were substantially up-regulated with the progress of on-tree fruit development both in the peel and in the flesh, registering the highest values at the last stage for both tissues (**Figure 3.5**). Notably in flesh tissue, a 8.1- and 11.0- fold increase of *CYCB* and *BCH* transcripts during the last stage compared to the initial stage was recorded, respectively. *PSY1* presented statistically significant increases at the S3, S4 and S6 stages of the peel and throughout all stages in the flesh (S2-S6); the most prominent gene regulation in the flesh was found at the S3 and S5 stages.

Contrarily, *CRTISO, LCYE, ECH and VDE* demonstrated overall a down-regulation expression pattern in both peel and flesh tissue. *CRTISO* was down-regulated at stages S3-S5 in peel; a similar trend was monitored in the flesh (S3-S6 stages). *ECH* had similar expression pattern (suppression) for both peel and flesh from stage S3 onwards. *LCYE* was down-regulated in peel throughout the five developmental stages; the most abundant decrease (128 fold change) was registered at the S5 stage. Interestingly, in flesh the decrease was registered at the S2 stage and remained undetected thereafter. *VDE* expression levels were repressed throughout S3 to S6 stages in peel and throughout all developmental stages in flesh. In both peel and flesh, *VDE* presented a substantial suppression at the S5 stages (49.5 and 36.8 fold change, respectively) compared to S1 stage.

Notably, *PSY1*, known to catalyse the first step in the carotenoid formation (Mendes et al., 2015) expression levels depicted a general up-regulation with the progress of on-tree fruit development both in the peel and in the flesh; however the highest transcript values in flesh tissue were monitored at S3 and S5 stages, not concomitant with total carotenoid accumulation (**Figure 3.5**). In another fleshy fruit (apple), Ampomah-Dwamena et al. (2012) also postulated that *PSY1* expression levels had no direct correlation with carotenoid content in different genotypes. On the other hand, *PDS*, an upstream pathway gene, presented statistically significantly higher transcript levels only in the peel at the last developmental stage (S6); *PDS* expression levels have been correlated with high- and low- carotenoid content apple cultivars (Ampomah-Dwamena et al., 2012).

Trans-lutein presented appreciably high accumulation in the first four stages in the peel with a reduction at ripe and over-ripe stages, while detectable amounts in the flesh were registered only during the initial developmental stages (S1, S2), as chloroplasts began to develop into chromoplasts. This decrease can be attributed to the fact that (1) *LCYE* was markedly down-regulated throughout the developmental stages in both peel and flesh compared to corresponding per tissue immature stage (notably not detectable transcripts during S3-S6 of flesh was monitored), (2) *LCYB* was down-regulated over the last developmental stages in flesh (S4-S6) and (3) *ECH* mRNA expression was generally down-regulated both in the peel and in the flesh (S3-S6). Fu et al. (2012) noted that lutein is showing a transient decrease in the flesh of loquat cultivars with the progress of on-tree fruit development, whereas there is no connection with *BCH* expression which appears to be up-regulated especially in the red-fleshed cultivar ‘Luoyangqing’. An upregulation of *BCH* gene expression was also monitored in our study in a similar flesh-type loquat cultivar. Ampomah-Dwamena et al. (2012) showed that *LCYE* expression was highly correlated with the carotenoid content in apple fruit skin, but that was not the case with the flesh; suggesting that down-regulation of *LCYE* is consistent with lower *trans*-lutein concentrations in flesh, in accordance with the findings of the current study.

Contrarily, the high *BCH* expression values registered in the flesh at the last two stages (S5 and S6) can be linked with the transient carotenoid accumulation of *trans*-β-cryptoxanthin in the last stages, concomitant with higher mRNA expression of *CYCB*

and non-detectable *LCYE* transcripts (stages 3-6). These findings are in accordance with Fu et al. (2012), where the *BCH* values for the red fleshed cultivar 'Luoyangqing' were drastically increased at the breaker stage in addition with the *CYCB* that was also higher after the S4 stage. Kato (2012) stated that the decrease of *LCYE* gene expression is related with the accumulation of β-carotenes in citrus fruits as the ε,β branch of the carotenoid pathway shifts to the β,β branch during transition from green to mature stage. Zhao et al. (2011) found that *BCH* is responsible for high β-cryptoxanthin content in persimmon fruit, in accordance with findings in other loquat cultivars (Zhang et al., 2016). The latter study suggests a direct link between the synthesis and accumulation of β-cryptoxanthin and the abundant expression of *BCH*. The transient increase of *trans*-β-carotene towards the S6 peel stage can also be linked with the up-regulation of *CYCB* and down-regulation of *LCYE*, as elsewhere described (Fu et al., 2012). Zhang et al. (2016) also links the higher β-carotene level in loquat peel with the abundant increase of *PSY1*, as well as *CYCB* and *BCH* mRNA expression levels.

VDE expression which leads to violaxanthin biosynthesis is significantly suppressed in almost all stages, both in the flesh and in the peel compared with the calibrator (S1 stage) (thus expecting very little violaxanthin); in the case of *ZEP*, which converts violaxanthin back to precursor molecules such as zeaxanthin, the main trend is that it is induced in several stages in the flesh. This is in accordance with metabolite levels, as both *cis*- and *trans*-violaxanthin are at appreciable low concentrations and/or non-detectable during several stages in the flesh (**Figure 3.4; Figure 3.5; Supplementary Table S5**).

3.5 Conclusions

The carotenoid profile of 'Obusa' fruits, an orange-fleshed loquat cultivar, was elucidated during distinct on-tree developmental stages. Results indicated that carotenoid composition was greatly affected during fruit development, revealing evident differentiations between flesh and peel tissue. The major carotenoids were *trans*-lutein and *trans*-β-carotene in peel, and *trans*-β-cryptoxanthin, *trans*-β-carotene, and 8-epoxy-β-carotene in the flesh. The presence of *cis*-lutein, citranaxanthin and 5,8-epoxy-β-carotene have not been reported previously in loquat, but only in other fruits of tropical

origin (De Rosso and Mercadante 2005; Zanatta and Mercadante 2007). Furthermore, a link was attempted to be established between gene up- or down-regulation during the developmental stages of the loquat fruit, and how their expression affects carotenoid content. Elevated content of *trans*-β-carotene both in the flesh and in the peel with the progress of on-tree fruit development can be linked with the up-regulation of *CYCB, a* main biosynthetic gene. Notably, the non-detectable amounts of *trans*-lutein in the flesh during the S3-S6 stages can be linked with the significant suppression of *LCYB* and *LCYE* expression levels during these stages. Transcripts levels of the latter gene were also significantly reduced throughout all developmental stages in the peel compared to the immature stage.

4 Dissection of the incidence and severity of purple spot physiological disorder in loquat fruit through a physiological and molecular approach

4.1 Abstract

The market potential of loquat (*Eriobotrya japonica*) is affected by the incidence and severity of purple spot (PS), a pre-harvest physiological disorder, evident as skin discoloration with depressed surface. Despite its economic impact, this disorder is still poorly understood and, to our knowledge, this is one of the few studies that aim to investigate possible mechanisms underlying such symptoms. The intensity and severity of PS in three loquat cultivars (‘Morphitiki’, ‘Karantoki’ and ‘Obusa’) was initially monitored during successive on-tree developmental stages. ‘Obusa’ fruits showed the highest incidence (58.6%), while ‘Morphitiki’ fruits did not show any symptoms. ‘Karantoki’ fruits demonstrated an intermediate response, with 31.3% of the fruit being affected. Thereafter, fruits with 30-50% severity were collected and used for further analysis; peel tissue was removed from both symptomatic and asymptomatic tissue of the same fruit for all examined cultivars. ‘Karantoki’ fruit peel with PS manifested the highest accumulation of total soluble sugars, sucrose, glucose and fructose contents, while in asymptomatic ‘Obusa’ fruit the accumulation of these metabolites registered the lower values, exception made for the sucrose. Furthermore, the involvement of polyphenolic (*PAL1*, *PAL2* and *PPO1*) and carbohydrate (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) biosynthesis transcripts in the incidence of PS was investigated. Results indicated that the induction of *CWI3* and *VI* expression levels in correlation with the catabolism of sucrose to glucose were highly linked with the incidence of PS appearance, highlighting genotype-specific responses and suggesting their potential use as markers in purple spot detection.

Keywords: *Eriobotrya japonica*, purple spot, preharvest disorder, sucrose, glucose, fructose, polyphenolic biosynthesis

4.2 Introduction

Loquat (*Eriobotrya japonica* Lindl) is a subtropical evergreen tree crop that is nowadays considered an important fruit with growing interest (Gisbert et al., 2009). Fruits ripe during early spring period (Badenes et al., 2013), when there is a lack of availability of other fresh commodities (Hadjipieri et al., 2017). However, the marketability of loquat is severely affected by the development of purple spot (PS), a preharvest physiological disorder that leads to skin discoloration. This disorder mainly affects the fruit surface exposed to the sun, heavily compromising general fruit appearance and rendering them unacceptable for marketing (Gariglio et al., 2002; Goulas et al., 2014). Gariglio et al. (2002) postulated that purple spot initiates at the deepest rind cells of the fruit, causing cellular dehydration and material deposition between the shrunken cytoplasm and the cell wall.

The severity of PS symptoms may substantially vary from year to year. Early harvest favors the development of PS compared with late harvest **(**Gariglio et al., 2003a). This is consistent with reports about the existence of environmental factors responsible for the onset of PS appearance in loquat fruit, such as minimum daily temperature and direct exposure of fruit to sun at breaker stage. However, to what extent such factors affect PS appearance still remains unknown. A positive correlation between PS appearance and fruit flesh sugar concentration has been proposed, since fruit thinning led to higher incidence of the disorder (Gariglio et al., 2003b). In addition, low temperature during the night and sun exposure also increased the gradient of total sugar concentrations in favor of the flesh compared with the rind, thus increasing the incidence of PS (Gariglio et al., 2008a). A companion study from the same group showed that there is a correlation between PS occurrence on loquat fruit and the alteration of water relations between flesh and peel at the fruit color break (Gariglio et al., 2008b). In summary, the higher PS incidence has been attributed to the simultaneous occurrence of flesh high sugar accumulation, the high growth rate enhanced by fruit thinning and environmental factors such as low night temperatures and direct sunlight exposure.

Soluble sugars, including sucrose (Suc), fructose (Fru) and glucose (Glu), are widely considered as major transcriptional regulators of many metabolic processes and

defense mechanisms **(**Li et al., 2012). Fruit growth and development relies on Suc supply that is considered necessary for fruit development and has been determined as a major index in harvesting fruits. Thus, Suc transport to the fruits is essential. Briefly, when Suc is transported into sink cells (fruit, root, etc.) a conversion to Fru and Glu follows by *neutral invertase* (*NI*) (Fotopoulos, 2005; Li et al., 2012) or to UPD glucose by *sucrose synthase* (*SuSy*) (Zhou et al., 2006). In turn, Glu and Fru are then phosphorylated to glucose-6-phosphate and fructose-6-phosphate by hexokinase (HXK) and fructokinase (FRK). The pathway involves Suc transport through the apoplast via Suc transporters or hydrolysis to hexoses by *CWI* or *SuSy* and subsequent uptake by monosaccharide transporters (Nguyen-Quoc and Foyer, 2001; Li et al., 2012; Wang et al., 2015). Special transporter proteins located in the vacuole membrane transport most of the Suc, Fru, Glu and other soluble sugars that have not been metabolized in the vacuole. *Vacuolar invertase* (*VI*) converts Suc to Fru and Glu. Hexose transporters transport the converted Glu and Fru [derived from Suc by *cell-wall invertase* (*CWI*)] into the parenchyma.

A similar discoloration to PS has been also observed in other Rosaceae fruit crops, such as apple and pear, known as superficial scald. This disorder is a chilling injury phenomenon, and discoloration occurs after extended periods of low-temperature storage (Lurie and Watkins, 2012), whereas PS, based on empirical data of our group, is mostly linked with low-temperatures during the growing season. In the case of apple, the discoloration is shown as a different browning limited to the skin tissue and the underlying cell layer.

The aim of the current study is to dissect potential mechanisms that regulate the development of PS in loquat fruit with the investigation of an array of morphological parameters along with expression levels of key genes that are involved in polyphenolic biosynthesis (*PAL1*, *PAL2* and *PPO1*) and carbohydrate biosynthesis (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*). These pathways appear to be either directly linked to PS occurrence, based on existing literature (carbohydrate metabolism), or potentially share a similar genetic basis with other physiological disorders, with similar symptoms, such as superficial scald (polyphenol metabolism).

4.3 Materials and Methods

4.3.1 Fruit material

Trees with contiguous vigor and canopy uniformity of three cultivars (‘Morphitiki’, ‘Karantoki’, ‘Obusa’) were used in the current study. Upon fruit set and immediately after fruit thinning, length and width from ten fruits per cultivar were measured at 7-day intervals for twelve weeks with the use of an electronic caliber (IS11112, Insize). ‘Karantoki’ and ‘Obusa’ fruits with PS severity of 30-50% at fully-ripe stage were selected for carbohydrate determination and gene expression analysis. Sugars and total RNA were extracted from peel tissue (both asymptomatic and symptomatic parts from the same fruit) of three biological replications per cultivar. In the case of ‘Morphitiki’ fruits that were free of PS disorder, only asymptomatic peel tissue was excised. All peel tissues were immediately frozen in liquid nitrogen and kept at – 80 °C until further analyses.

4.3.2 Methodology

At the commercial maturity stage, total harvested fruits per cultivar were rated into five distinct groups according to the severity incidence of the PS appearance as described in §2.3. Peel sugars (TSS, Suc, Glu, Fru) were extracted using the method described by Jin et al. (2007) with slight modifications as described in §2.2.4. The expression levels of genes involved in polyphenolic biosynthesis (*PAL1*, *PAL2* and *PPO1*) and carbohydrate biosynthesis (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) were determined as illustrated in **Figure 4.1**. Extraction was carried out based on a modified cetyltrimethylammonium bromide (CTAB) protocol originally developed by Gambino et al. (2008) as described in §2.8. RNA integrity was spectrophotometrically assessed (Nanodrop 1000 Spectrophotometer, Thermo Scientific) and visually inspected with gel electrophoresis. Samples were then treated with RNase-free DNase (Cat. No. NU01a, HT Biotechnology LTD, England) to remove total gDNA, as elsewhere described (Georgiadou et al., 2016). PrimeScript^{TM} RT reagent Kit (Takara Bio, Japan) was used to reverse transcribed total RNA (0.5 μg), according to the manufacturer's protocol (Takara Bio, Japan). Subsequently,

quantitative real-time RT-qPCR was carried out as described in §2.9 with BioRad IQ5 real-time PCR cycler (BioRad, USA).

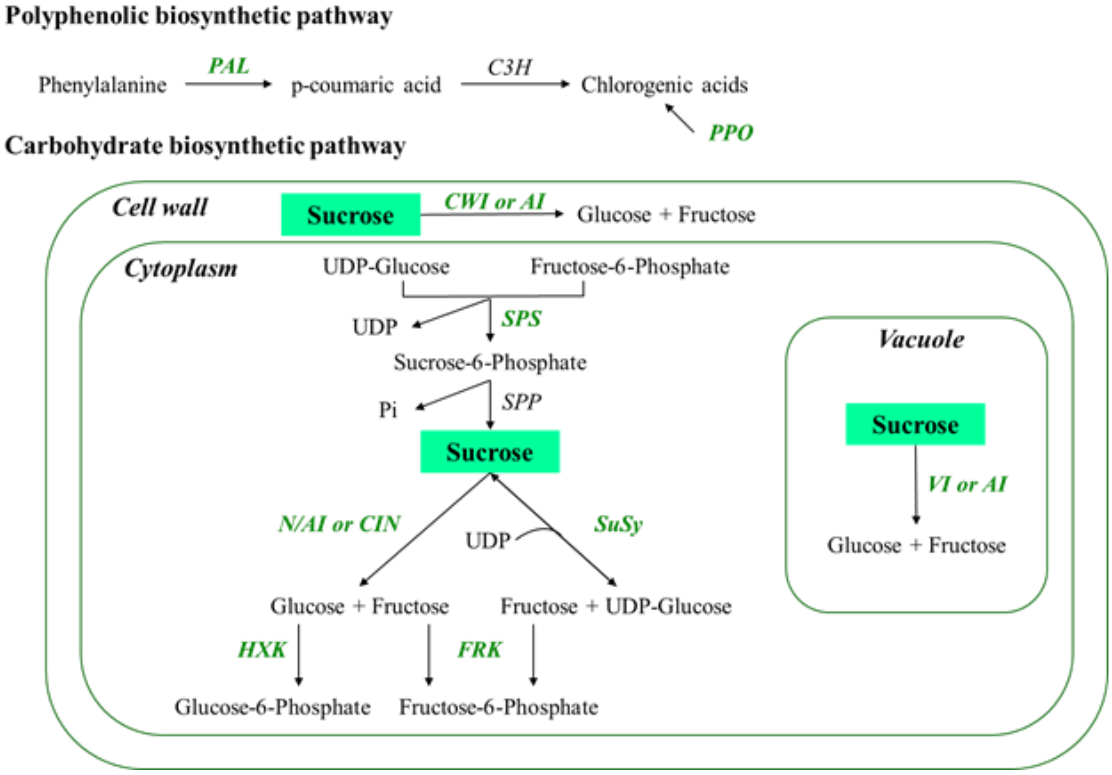

Figure 4.1: Polyphenolic and carbohydrate biosynthetic pathways. Figure is modified from Nookaraju et al., 2010; Li et al., 2012; Busatto et al., 2014.[12]

Malus domestica-specific primers for transcripts *CWI2*, *CWI3*, *NI2*, *NI3*, and *SuSy* were used as no *E. japonica*-specific primers have been previously reported nor are they available in public databases. The selection of *Malus* was based on its close relation with *Eriobotrya* (both members of the Rosaceae family) and wide availability of published sequences. These were crossed-referenced after performing bioinformatics analysis on the NCBI database[13] that resulted in 100% primer similarity.

[12] *Genes with expression levels studied are in italic green bold letters.The enzymes/genes are: PAL, phenylalanine ammonia lyase; PPO, polyphenol oxidase; CWI, cell wall invertase; AI, acid invertase; SPS, sucrose phosphate synthase; SPP, sucrose phosphate phosphatase; N/AI, neutral / alkaline invertase; CIN, cytoplasmic invertase; SuSy, sucrose synthase; HXK, hexokinase; FRK, fructokinase; VI, vacuolar invertase.*
The metabolites are: phenylalanine; p-coumaric acid; chlorogenic acids; UDP-glucose; fructose-6-phosphate; sucrose-6-phosphate; sucrose; glucose; fructose; glucose-6-phosphate.

[13] http://www.ncbi.nlm.nih.gov/

4.3.3 Statistical analysis

Statistical analyses were carried out by comparing the averages of the two cultivars (cvs. 'Karantoki' and 'Obusa') on both symptomatic (30-50%) and asymptomatic peel tissue at fully-ripe stage, as well as asymptomatic tissue from cv. 'Morphitiki' at fully-ripe stage, based on the analysis of variance (one-way ANOVA) according to Duncan's multiple way test with a significance level of 5% ($P \leq 0,05$), using the SPSS v.17.0 statistical analysis software package.

4.4 Results and Discussion

4.4.1 Incidence of purple spot

Fruit growth (length and width measurements) of all three examined cultivars followed a typical sigmoid curve (**Supplementary Figure S1**). Almost 60% of harvested 'Obusa' fruits presented PS symptoms rendering them unsuitable for commercial purposes (**Table 4.1; Figure 4.2)**. 'Karantoki' fruits with PS symptoms accounted for 31.6% of total harvested fruit. Contrarily, 'Morphitiki' fruits did not show any symptoms (**Table 4.1; Figure 4.3**). The SI was 0.51 and 0.12 for 'Obusa' and 'Karantoki' fruits respectively (**Table 4.1)**. The high PS incidence and severity of cv. 'Obusa', an orange-fleshed loquat cultivar (Hadjipieri et al., 2017), has led to the abandonment of this cultivar under Cypriot conditions.

However, for experimental purposes, this cultivar represents an excellent plant material for comparative purposes in order to decipher the abovementioned physiological disorder. 'Karantoki' and 'Morphitiki' are the most important commercially grown cultivars in Cyprus (Goulas et al., 2014, Hadjipieri et al., 2019; Hadjipieri et al., 2020).

Notably, 'Karantoki' is the most widely cultivated one, due to its earliest harvest and bigger size; however, the susceptibility to PS disorder that is particular evident in growing seasons with unfavorable climatic conditions indicates the need to reconsider cultivar selection for new orchard plantations. Simultaneously, advances in genomic studies that are extensively applied in other species within the Rosaceae family is expected to offer new insights in loquat breeding programs through marker-assisted selection (Badenes et al., 2013). Towards this aim, the current study is trying to shed

some light toward elucidation of sugar catabolism and selection of genes that can be putatively considered markers of purple spot severity.

Table 4.1: Purple spot incidence on the three different loquat cultivars 'Morphitiki**',** 'Karantoki' and 'Obusa' at fully-ripe stage. 'NFSI' stands for Number of Fruits with Severity Incidence.

		Cultivar		
		Morphitiki	**Karantoki**	**Obusa**
Purple spot incidence on fruit	**Severity incidence**	**%**	**%**	**%**
0-10%	NFSI-1	100%	68.7%	41.4%
10-30%	NFSI-2	-	19.4%	11.2%
30-50%	NFSI-3	-	9.6%	19.9%
50-70%	NFSI-4	-	2.2%	16.9%
>70%	NFSI-5	-	0%	10.6%
Total		100%	100%	100%
Severity Index		**0**	**0.12**	**0.51**

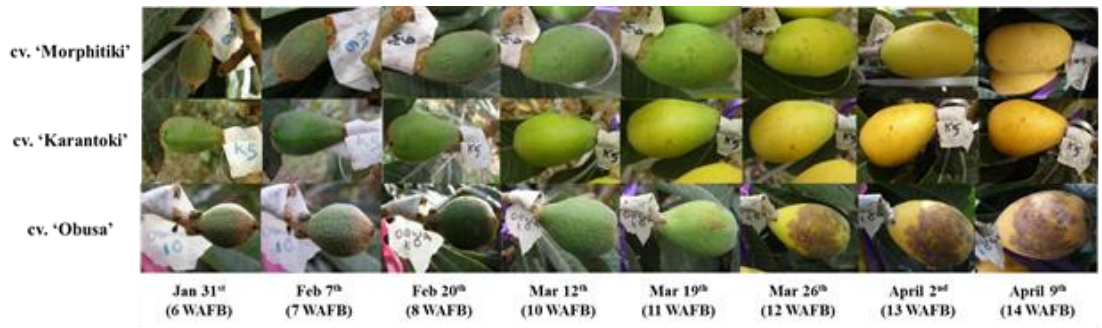

Figure 4.2: Purple spot incidence during successive on-tree developmental stages of the examined loquat cultivars (cvs. 'Morphitiki', 'Karantoki' and 'Obusa'). 'WAFB' stands for weeks after full bloom.

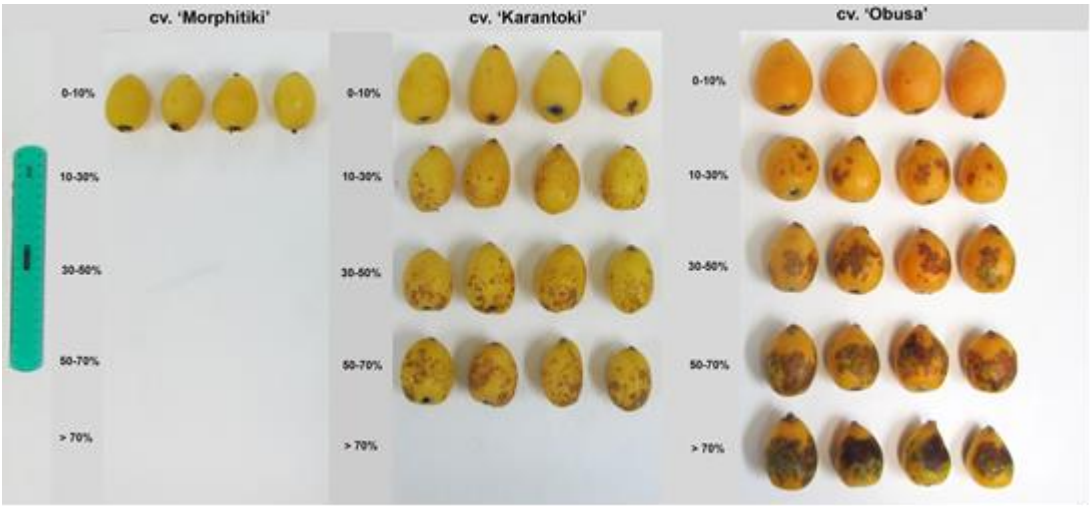

Figure 4.3: Severity Incidence (SI) categorization for purple spot appearance on cvs. 'Morphitiki', 'Karantoki' and 'Obusa' at fully-ripe stage.

4.4.2 Total soluble sugars (TSS), sucrose, glucose and fructose contents in the peel

Soluble sugars including Suc, Glu and Fru are known to act as key signal molecules regulating the expression of many genes involved in plant metabolic processes and defense responses, thus regulating plant growth and development (Li et al., 2012).

Total soluble sugars ranged from 54.9 to 105.8 mg^{-1} FW. Fructose (Fru) was the most abundant type sugar assessed with values ranging from 26.4 to 55.7 mg^{-1} FW. Sucrose and glucose accounted instead for 11.2–22.8 mg^{-1} FW and 12.4–20.0 mg^{-1} FW, respectively. Present results indicated that 'Karantoki' fruits with PS showed higher TSS, Suc, Glu and Fru contents compared with 'Obusa' (both asymptomatic and with PS symptoms) and 'Morphitiki' fruits. A slight increase in TSS, Glu and Fru contents for cv. 'Obusa' with PS was also noted in comparison with asymptomatic 'Obusa' fruit peel but, interestingly, Suc content was lower (**Figure 4.4**). This decrease can be attributed to the fact that invertases break down Suc to Glu and Fru, as illustrated in **Figure 4.2**. Carbohydrate content is a key factor to fruit quality during maturation. Numerous studies showed that, in ripe loquat flesh tissue samples, Glu and Fru are the dominant sugars (Song et al., 2016; Cai et al., 2019). Gariglio et al., (2008a) linked sugar accumulation in the peel, at the color break stage, with the PS incidence with peel

tissue from fruit grown indoors exhibited higher carbohydrate content (TSS, Suc, Fru and Glu) and reduced PS incidence in comparison with affected peel tissue from fruit grown outdoors. In our study fruit peel with PS has higher carbohydrate content in comparison with fruit peel from the same fruits that were asymptomatic.

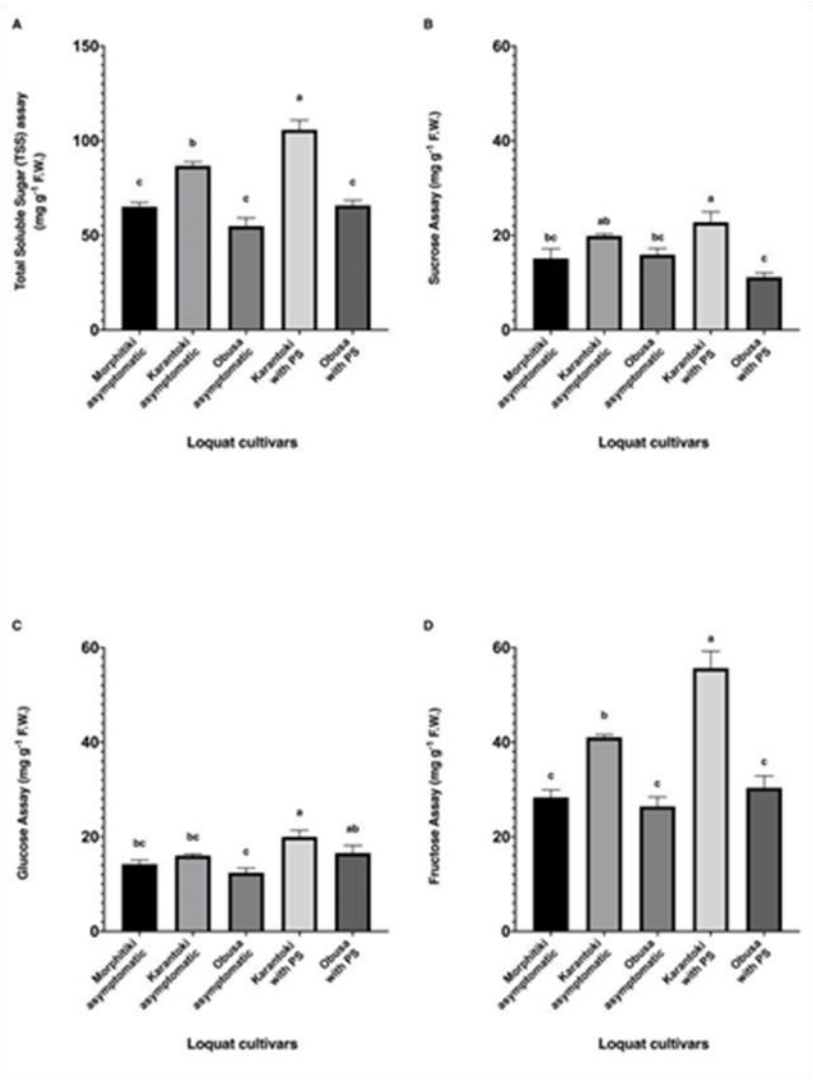

Figure 4.4: Total soluble sugar (A), sucrose (B), glucose (C) and fructose (D) contents at fully ripe stage in loquat fruit peel of cvs. 'Karantoki', 'Obusa' and 'Morphitiki', on both symptomatic (30-50%) and asymptomatic peel tissue of the same fruits at fully-ripe stage.[14]

[14] Values followed by the same letter are not significantly different according to Duncan's multiple range test at significance level 5% ($P \leq 0.05$). Data are the means of three biological replications ± SE.

4.4.3 Gene expression profiling

Thirteen key transcripts implicated in the polyphenolic and carbohydrate biosynthesis pathways were examined for all three examined cultivars. In particular, relative expression levels of polyphenolic biosynthetic genes (*PAL1, PAL2* and *PPO1*) and carbohydrate biosynthetic genes (*CWI2, CWI3, SPS1, SPS2, NI2, NI3, SuSy, HXK, FRK* and *VI*) in loquat fruit peel asymptomatic and symptomatic tissue were monitored and their regulation compared with the house keeping reference gene (*ACT*) is depicted in the form of a heat map **(Figure 4.5**).

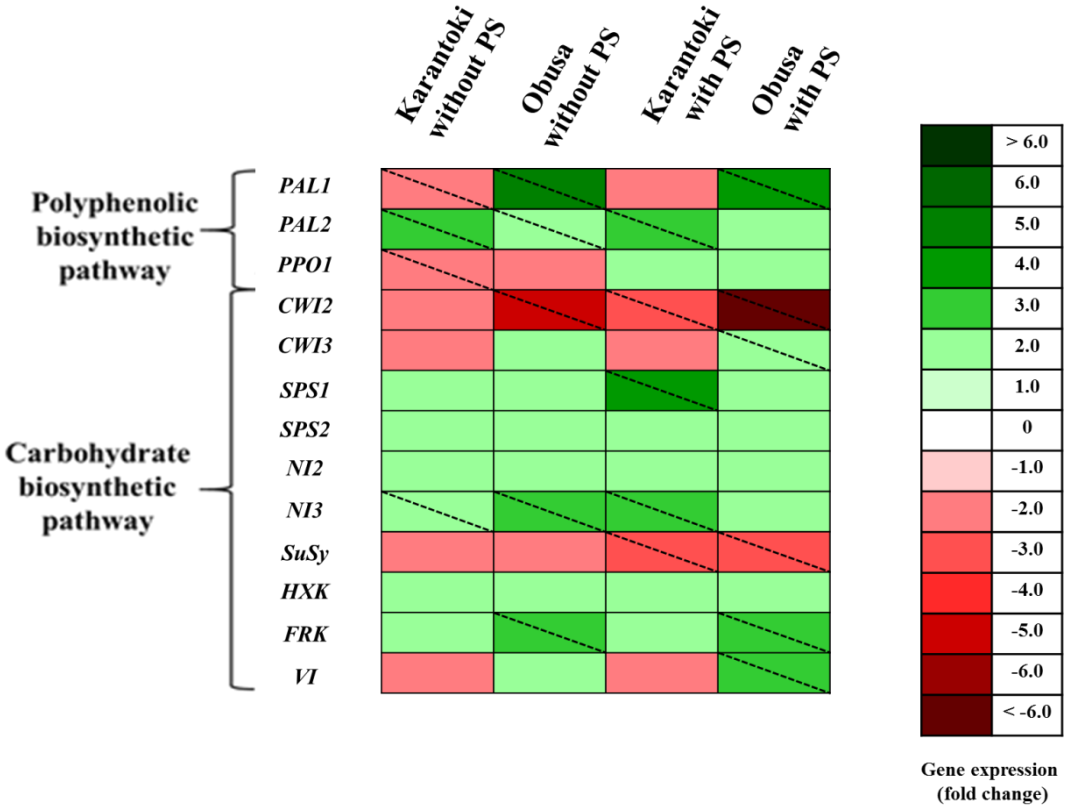

Figure 4.5: Relative expression levels of polyphenolic biosynthetic genes (*PAL1, PAL2* and *PPO1*) and carbohydrate biosynthetic genes (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) in loquat fruit peel tissue of cvs. 'Karantoki' and 'Obusa', on both symptomatic (30-50%) and asymptomatic peel tissue at fully-ripe stage (n = 3).[15]

[15] *Relative mRNA expression levels were evaluated by real-time RT-qPCR using three biological repeats. A diagonal line in a box indicates a statistically significant value ($P \leq 0.05$). A scale of colour intensity is presented as a legend. Up-regulation is indicated in green; down-regulation is indicated in red. Relative gene expression values were determined using cv. 'Morphitiki' asymptomatic peel tissue at fully-ripe stage as a calibrator.*

With regard to the polyphenolic biosynthesis pathway, *PAL1* was up-regulated in both symptomatic (3.4-fold) and asymptomatic (4.0-fold) 'Obusa' fruits. *PAL1* was down-regulated in 'Karantoki' asymptomatic and symptomatic fruit by -1.3 and -1.6-fold, respectively. No genotype-dependent trend in regulation of *PAL1* was observed, suggesting that it is not linked with PS incidence. Gene induction was noted for *PAL2* for symptomatic 'Karantoki' tissue that registered a 2-fold increase. However, *PAL2* induction cannot be linked with PS as similar up-regulation was found in cv. 'Obusa' with PS.

Shan et al. (2008) revealed that *PAL1* was more highly expressed in mature fruit in cvs. 'Luoyangqing' and 'Baisha' in comparison with *PAL2*. This suggests that *PAL2* might be more tightly linked with the phenylpropanoid synthesis during early development compared with *PAL1* that might be more correlated with flavonoid biosynthesis and lignification of mature fruit. Therefore, the two *PAL* alleles are expressed differentially during development, supporting our findings in regard with the 'Obusa' cultivar.

PPO1 registered a 1.3- and a 1.6-fold increase for cvs. 'Karantoki' and 'Obusa' with PS, respectively, whilst it was down-regulated by 1.9- and 1.3-fold for the same cultivars lacking PS symptoms. Interestingly, similar symptoms have been recorded in other Rosaceae fruits (apple and pear), evident as superficial scald (a chilling post-harvest disorder) that is manifested through diffuse browning limited to the skin and the underlying six cell layers. Whilst PS appears on-tree, scald development is a disorder that occurs after extended cold storage regimes. An accumulation of chlorogenic acid (as a consequence of the activation of *PAL*) seems to be the reason for the browning coloration and a polyphenol oxidase gene (*PPO*) is the cause for its further oxidation in apple (Busatto et al. 2014). However, warm and dry preharvest weather conditions increase the intensity of superficial scald post-storage, while low night temperature before harvest as well as during days with temperatures less than 10°C lead to less severe incidence of the disorder (Lurie and Watkins, 2012). Current findings do not support the original assumption that *PPO* might have a direct link with PS appearance, although higher intensity of PS disorder appearance has been monitored in loquat orchards during cold winter months [January, February (Hadjipieri, unpublished data)] at BBCH stage 709 and before stage 805 (Martinez-Calvo et al., 1999).

NI2 and *HXK* demonstrated small increase in expression levels for both asymptomatic or with PS symptoms cultivars (‘Karantoki’ and ‘Obusa’) in comparison with ‘Morphitiki’. *NI3* had a 2.5-fold increase for cv. ‘Karantoki’ with PS, but only 1.2-fold increase for cv. ‘Obusa’ with PS. Increased *NI* transcript levels are linked with metabolism of Suc to Glu and Fru, which might also explain the increased Fru levels in the cultivars with a noted difference in cv. ‘Karantoki’ with PS. On the other hand, *SuSy* had a significant down-regulation for both cvs. ‘Karantoki’ and ‘Obusa’ with PS of 2.2- and 2.4-fold, respectively. The down-regulation of *SlSuSy* as well as *SlLin5* (*CWI*) was linked to higher sucrose content in tomato fruits that were treated with xenobiotics (Christou et al., 2019).

Increased expression of *SPS1* and *SPS2* for both cvs. ‘Karantoki’ and ‘Obusa’, with PS or asymptomatic peel tissue, reveal that soluble sugars were produced via biosynthesis of Suc followed by hydrolysis into Glu and Fru. A sharp rise for both *SPS1* and *SPS2* was also noted by Song et al. (2016), explaining that the increases in the expression levels of these two genes suggested that soluble sugars were produced through synthesis of Suc followed by hydrolysis into Glu and Fru.

Fructokinase has an important role in the utilization of Fru (Qin et al., 2014). In the current study, *FRK* showed an increase in expression which is not in accordance with the high Fru content recorded, especially in cv. ‘Karantoki’ with PS (**Figure 4.4**). Similarly, Qin et al. (2014) postulated that although the expression of *FRK* decreased with progressing fruit maturation, Fru levels were higher.

Acidic invertase expression that involves *CWI3* and *VI* is up-regulated in ‘Obusa’ samples with PS. These two genes demonstrate strong correlation with PS appearance in cv. ‘Obusa’, which manifest the genotype significance in the appearance of the physiological disorder. *CWI3* can be considered as the dominant isoform gene for loquat mature fruit, in comparison with *CWI2* that was down-regulated. The observed induction of *CWI3* and *VI* is more abundant in ‘Obusa’ samples with PS but showed no significant induction in ‘Karantoki’ samples with PS which might be supported by the severity of PS symptoms (**Supplementary Table S5; Figure 4.4; Figure 4.5**). This can also be linked with the catabolism of Suc to Glu with a possible correlation with PS appearance.

4.5 Conclusions

Low temperatures during the last on-tree developmental stages have been empirically linked with increased incidence and severity of PS disorder at the beginning of the harvesting season. Apart from climatic conditions, genotype possess a critical role in the incidence and severity of PS disorder. With the aim to shed some light on the incidence of PS disorder and to elucidate the genotype significance, thirteen genes involved in the polyphenolic and carbohydrate biosynthetic pathways in loquat fruit of the three examined cultivars with the same degree of symptoms were examined. Acidic invertase levels involving *CWI3* and *VI* were substantially up-regulated in the symptomatic tissue of 'Obusa' fruits; this cultivar registered the highest overall sensitivity to the disorder, even from the colour break stage. Overall, the induction of *CWI3* and *VI*, aligned with catabolism of sucrose to glucose, provide potential molecular and metabolic markers for the cellular detection of PS.

5 The impact of genotype and harvesting day on qualitative attributes, postharvest performance and bioactive content of loquat fruit

5.1 Abstract

Due to scalar on-tree ripening, harvest of loquat fruit is successive, spanning for several weeks, depending on the cultivar considered and the cultivation practices applied. Notably, early harvested fruit receive appreciably high prices on the market. The aim of the current study was to dissect the effect of harvesting day on mechanical properties, postharvest performance and phytochemical attributes (free and bound phenolic compounds and antioxidant capacity) of the predominant loquat cultivars grown in Cyprus (cvs. 'Karantoki' and 'Morphitiki'). Determination of the aforementioned attributes at harvest (H) and after additional maintenance at room temperature for 3 days (H+3) for four successive harvesting dates (H1-4) were determined. Flesh firmness was slightly higher in early-harvested compared to late-harvested fruit, while slight or no differences after shelf life period for both cultivars were monitored. 'Karantoki' fruits manifested higher values of ripening index (SSC/TA) than 'Morphitiki'; such values were higher with the progress of harvest date due to a significant decrease of titratable acidity. No evident differences were registered between the harvest date and the shelf life period for both cultivars regarding free phenolic content. This study also highlights the significance of bound phenolics that contribute to the phenolic fraction of loquat fruit for 21.6-37.5%, depending on the cultivar and storage condition applied. The current study additionally sheds light in the unexploited area of phytochemical properties of loquat fruits derived from successive harvesting dates. 'Morphitiki' fruits were characterized by higher free and bound phenolic contents, along with higher antioxidant capacity, registering the highest values during shelf life at the second harvesting day.

Keywords: *Eriobotrya japonica, harvest, phytochemicals, free phenolics, bound phenolics, antioxidant capacity*

5.2 Introduction

Loquat is a subtropical evergreen tree, originating from temperate China. Nonetheless, its global outspread started from Japan and since then it has undertaken diffusion in other markets (Tian et al., 2011). Its unique reversed annual cycle enables this species to satisfactorily adapt in the Mediterranean basin and other subtropical climates (González et al., 2010). The main countries of loquat production are China and Spain; the latter is the leading exporting country (Caballero and Fernández, 2004). In Cyprus, loquat cultivation is mainly favoured in the coastal regions. Despite its adaptability, a limited area is being cultivated, while ca. 30% of total area is covered by mesh and/or plastic to secure production volumes and advance fruit ripening. 'Karantoki' and 'Morphitiki' are considered as indigenous and/or traditional cultivars and are the most predominant, yet largely uncharacterised regarding their pomological properties (Goulas et al., 2014; Hadjipieri et al., 2019). Therefore, the evaluation of local germplasm and indigenous cultivars stands as an emerging need.

Loquat commercial production has risen in interest as it has stepped out from a small local cultivation to a niche product that receives appreciably high prices (Liu et al., 2016), particularly the early harvested fruit. This is mainly attributed to the lack of other fleshy fruits in the market during its harvesting period. Overall, loquat can potentially be identified as a promising fruit for the market, since traditional fruit crops in the Mediterranean climates are saturated (Chalak et al., 2014). However, its marketability is affected by purple spot appearance; a pre-harvest physiological disorder that leads to discoloration of the skin tissue without any effect in the inner fruit flesh. This disorder can affect up to 30% of the fruit's skin appearance, thus rendering them inappropriate for the market (Gariglio et al., 2002).

During fruit maturation, the physiology of loquat is evolving with the fruit size showing a rapid increase between the breaker (BBCH phenological stage 801 based on Martinez-Calvo et al. (1999) and half ripe stage (BBCH stage 805). The full colour is acquired at the ripe stage (BBCH stage 809) and based on the cultivar may differ from pale yellow to deep orange (Hadjipieri et al., 2017).

Loquat fruit remains rather unexploited regarding its phytochemical properties, since relatively few studies exist concerning its health-promoting benefits. Zhang et al.

(2015) found that hydroxycinnamic acid and flavonols are the main constituents of loquat phenolic fraction. The fluctuation of the total phenolic content of 'Mogi' fruits throughout successive fruit developmental stages were mainly linked with changes occurring in the chlorogenic acid concentrations (Ding et al., 2001). Furthermore, variations on the total phenolic content and the antioxidant capacity among different genotypes exist (Ercisli et al., 2012) that can partially be attributed to their flesh coloration (Zhang et al., 2015). Other studies identified cultivars with higher phenolic and flavonoid contents which can be promoted as of high nutritional value and thus beneficial to human health (Ferreres et al., 2009; Xu and Chen, 2011; Xu et al., 2014). However, such studies have monitored the total phenolic content without taking into account the contribution of bound phenolic content, that only recently its contribution was acknowledged. Concomitantly, there has been no investigation on the harvesting period and how it is affecting the fruit phytochemical properties. Su et al. (2014) postulated that phenolic compounds exist both in free and bound forms in the cells of the plants and in contrast with the free phenolic compounds the bound ones, localized in the cell wall area, cannot be extracted with water or aqueous/organic solvents mixtures.

The aim of the current study was to dissect changes on qualitative attributes of two indigenous loquat cultivars grown in Cyprus in correlation with the harvest period, through the evaluation of mechanical properties, qualitative attributes, postharvest performance and bioactive content, with special reference to both free and bound phenolic compounds.

5.3 Materials and Methods

5.3.1 Fruit material

Three trees per cultivar with contiguous vigor, shape, age and maturity stage were harvested at four successive commercial harvesting dates[16] for cv. 'Karantoki' and three for cv. 'Morphitiki' in 2016 as described in §2.1 based on fruit size and external color. Total yield per harvest as well as purple spot severity was registered.

[16] March 4th, March 18 th, April 1st and April 12 th, designated as H1, H2, H3 and H4, respectively.

Subsequently, homogeneous lots of 60 representative sound fruits (subdivided into two 30-fruit sublots) per cultivar and harvest period were selected for further analysis. Sublots were analyzed upon harvest and after maintenance for 3 days at room temperature (shelf life), respectively. Flesh tissue was flash frozen, homogenised to fine powder with liquid nitrogen and kept at -80°C until needed for phytochemical analysis.

5.3.2 Methodology

Weight, color parameters (L*, a*, b*, Chroma and hue angle), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) were determined as described in §2.2. Ripening index (RI) was calculated as the SSC/TA ratio. Free and bound phenolics were extracted and determined according to the protocol described in §2.5. DPPH scavenging capacity and the ferric acid reducing antioxidant power (FRAP) were determined according to the protocol previously described in §2.6.

5.3.3 Statistical analysis

Statistical analyses [Two-way ANOVA and Duncan Post-Hoc at a 5% significance level ($P \leq 0.05$)] for weight loss, soluble solids content, titratable acidity, ripening index, flesh firmness as well as phenolics, DPPH and FRAP (free and bound form) were carried out using the software package SPSS v22.0 (SPSS, Inc., Chicago, IL, USA).

5.4 Results and Discussion

5.4.1 Quality attributes

As the harvest period was progressing, the fruit size for both cultivars was increasing with cv. ‘Karantoki’ registering higher mean weight values. Interestingly, almost half production (46%) of cv. ‘Morphitiki’ was harvested in a single harvesting day (H3) that can be considered as a comparative advantage of the cultivar. Early harvested ‘Karantoki’ fruits, which accounts for the 9 % of the total yield, receive appreciably higher prices compared the other harvest dates. Total yield was similar for both cultivars

with the higher yields being registered at the last two harvest dates (65.7% for 'Karantoki' and 81.6% for 'Morphitiki' cultivar, **Table 5.1**).

'Morphitiki' presented lower weight loss (3.8-6.7%) compared to 'Karantoki' (5.0-6.9%) fruit subjected to 3 d shelf life. Notably, weight loss values were lower during the last harvesting time (H4) for both cultivars (data not shown). Similar results have been also postulated by Guerra et al. (2009) in plum fruit, where late harvested fruit were characterised by less weight loss than fruit from early harvest.

Table 5.1: Price range at farmer, wholesale and retail level, total yield (kg) and its proportion (%) per harvest of 'Karantoki' and 'Morphitiki' fruits.

	Price (€)**			**Yield (kg, %)**	
Harvest*	**Farmer**	**Wholesale**	**Retailer**	**Karantoki**	**Morphitiki**
H1	6.0	7.5	9.0	20.7 (9.0)	-
H2	5.0	6.0	7.0	58.4 (25.3)	45.9 (18.4)
H3	4.5	5.0	6.5	47.2 (30.7)	114.5 (46.0)
H4	3.0	4.0	5.0	81.0 (35.0)	88.7 (35.6)

**H1, H2, H3 and H4 represent the harvest dates (March 4th, March 18th, April 1st and April 12th) of loquat fruits.*

***Prices at farmer, wholesale and retail level are similar for both cultivars.*

A 3.5-5.6% of 'Karantoki' fruits per harvest were classified as unmarketable due to the incidence of purple spot disorder, while 'Morphitiki' fruits were free of symptoms (data not shown). Over the harvest time period, PS appearance is lessened, in accordance with earlier studies (Gariglio et al., 2002, 2008) which pointed out that early-maturing orchards had higher incidence of purple spot at the beginning of the season. Gariglio et al. (2002) also indicated that the effect of low temperature, especially in early-ripening cultivars, can be the main co-factor responsible for the PS appearance on the fruit. In our study, the relatively low incidence of purple spot towards the last harvesting days can be attributed to the warm climate conditions.

Table 5.2: Soluble solids content (SSC), titratable acidity (TA), ripening index (RI), flesh firmness (FF) values for 'Karantoki' and 'Morphitiki' fruit, at successive harvests and after additional maintenance at room temperature for three days.

Treatment	SSC (°Brix)	TA (Malic acid, %)	RI	FF (N)
cv. 'Karantoki'				
H1*	11.4±0.1e**	0.69±0.04a	16.4±1.1fg	1.22±0.05ab
H1+3d	11.9±0.6de	0.39±0.03c	31.3±4.0de	1.18±0.06abc
H2	12.0±0.1cde	0.32±0.01cd	37.3±0.9cd	1.15±0.05abc
H2+3d	12.5±0.4abcd	0.23±0.02de	54.3±5. 3b	1.17±0.04abc
H3	12.0±0.2cde	0.30±0.00cd	39.6±0.2cd	1.09±0.03cd
H3+3d	12.9±0.4abc	0.18±0.00e	70.1±1.3a	1.12±0.04bcd
H4	12.4±0.5bcd	0.27±0.02de	45.1±2.6c	1.01±0.05de
H4+3d	13.3±0.2ab	0.24±0.03de	54.8±6.9b	0.91±0.02e
cv. 'Morphitiki'				
H2	12.0±0.1cde	0.75±0.04a	16.1±0.8g	1.19±0.04abc
H2+3d	13.3±0.3a	0.54±0.04b	24.8±1.7egf	1.24±0.05a
H3	12.3±0.2cde	0.52±0.05b	23.4±2.5efg	1.12±0.03bcd
H3+3d	12.7±0.2abcd	0.31±0.02cd	40.8±1.9cd	1.13±0.03abc
H4	12.3±0.2cde	0.49±0.06b	25.3±2.8ef	1.08±0.04cd
H4+3d	12.8±0.3abcd	0.34±0.01cd	38.0±2.0cd	1.00±0.03de

**Different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).*

*** Results are the means ± standard error (n=3 for SSC, TA and RI, n=30 for FF).*

Flesh firmness (FF) slightly decreased with the progress of on-tree ripening for both cultivars ranging from 1.01 - 1.22 N (H) and 0.91 - 1.18 N (H+3) for 'Karantoki' and 1.08 – 1.19 N (H) and 1.00 - 1.24 N (H+3) for 'Morphitiki', respectively. However, advanced harvest followed by shelf life (H4+3) led to flesh softening for both cultivars (ca. 0.9 N for 'Karantoki' and 1.0 N for 'Morphitiki' fruits) while this was not the case among the rest harvesting and shelf life periods (**Table 5.2**). Extended storage of loquat fruits is also not desirable as for certain loquat cultivars may lead to further firmness reduction (Amorós et al., 2008). Previous study indicated that cv. 'Morphitiki' was characterized by firmer fruit with appreciably acceptable appearance in comparison with 'Karantoki' (Hadjipieri et al., 2019). Soluble solids content (SSC) slightly increased

with the progress of harvesting day for ‘Karantoki’ cultivar (from 11.4 ^{o} Brix at H1 to 12.4 ^{o} Brix at H4), while titratable acidity (TA) markedly decreased for both cultivars. ‘Morphitiki’ fruits had higher TA values compared with ‘Karantoki’ fruits for all harvests. The dramatic decrease of TA during fruit’s shelf life was also indicated in previous studies (Cao et al., 2009; Goulas et al., 2014; Hadjipieri et al., 2019). The ripening index (RI) varied significantly both between cultivars and among the successive harvesting days ranging from 16.4-45.1 and 16.1-25.3 at harvest up to 31.3-70.1 and 24.8-40.8 during shelf life for ‘Karantoki’ and ‘Morphitiki’ fruits, respectively. Despite the fact that loquat fruit is considered as a non-climacteric type fruit (Tian et al., 2011), substantial changes on qualitative attributes occurs that are not necessarily linked with fruit deterioration.

Color parameter a* had higher values after the shelf life period with more evident differences in ‘Morphitiki’ fruits. The a*/b* ratio also registered higher values after the three days shelf life, manifesting deeper fruit color compared with the freshly harvested fruits. The L* colour parameters were higher at harvest for ‘Morphitiki’, as a more luminous cultivar compared to ‘Karantoki’.

Colour saturation, based on Chroma and hue angle parameters, showed slight variations although the hue angle was higher at harvest for both cultivars compared with the corresponding values during shelf life periods. ‘Karantoki’ had higher hue angle values (87.1±0.3 for H3 and 87.8±0.3 for H4) in comparison with ‘Morphitiki’ (82.9±0.6 for H3 and 83.7±0.5 for H4); this can be partially explained considering that ‘Karantoki’ peel is yellow and ‘Morphitiki’ is orange (**Table 5.3)**. Furthermore, the higher a*/b* ratio values of ‘Morphitiki’ is an indicator of higher coloration.

All aforementioned quality parameters can be used as indicators for harvesting loquat fruit (Pinillos et al., 2011; Cañete at al., 2015). In the current study, early harvest coincided with higher TA values and lower RI, while non-significant differences in FF were monitored (**Table 5.2**). Pinillos et al. (2011) reported that a minimum SSC value of 10 ° Brix, along with a preferable colour as harvest indicators while Besada et al. (2013) mentioned a SSC value of 11 ° Brix to ensure optimum consumer satisfaction. In our study, SSC values for both cultivars were well above the threshold value of 11 ° Brix. The RI registered elevated values after shelf life, particularly at advanced harvestings for both cultivars (**Table 5.2**). Overall, the harvesting period is of great

importance as early harvest may lead to disordered sweet/acid equilibrium with the outcome of harvesting over-acid fruits; a late harvest can also lead to absence of acidity which results to poor fruit quality (Besada et al., 2013).

Table 5.3: Color parameters of 'Karantoki' and 'Morphitiki' fruits at successive harvests and after additional maintenance at room temperature for three days.[17]

	Color parameters					
Treatments	**L***	**a***	**b***	**a*/ b***	**Chroma**	**Hue angle**
cv. 'Karantoki'						
H1*	60.1±1.0 f**	2.2±0.4g	46.0±1.5bc	0.05±0.01h	46.1 ±1.5bc	86.7±0.4ab
H1+3d	59.8±0.6f	3.6±0.4f	44.5±0.5cd	0.08±0.01fg	44.7±0.5cd	85.4±0.5c
H2	59.2±0.8f	3.4±0.4f	45.6±0.5cd	0.08±0.01g	44.8±0.5cd	85.7±0.5bc
H2+3d	63.0±0.4cd	4.9±0.3de	47.3±0.4ab	0.1±0.00ef	47.5±0.4ab	84.1±0.3de
H3	60.2±8f	2.2±0.2g	43.7±0.5d	0.05±0.01h	44.7±0.5d	87.1±0.3a
H3+3d	64.5±0.4bc	4.1±0.2ef	47.4±0.4ab	0.09±0.01fg	47.6±0.4ab	85.1±0.3cd
H4	63.5±0.7bcd	1.4±0.3g	43.6±0.5d	0.03±0.01h	43.6±0.5d	87.8±0.3a
H4+3d	60.3±0.6f	3.3±0.3f	42.8±0.5d	0.08±0.01g	42.9±0.5d	85.5±0.3bc
cv. 'Morphitiki'						
H2	65.0±0.3ab	6.7±0.3a	48.2±0.4a	0.14±0.01cd	48.8±0.4a	81.9±0.4fg
H2+3d	61.0±0.5ef	8.3±0.3a	46.8±0.5ab	0.18±0.01a	47.5±0.5ab	79.9±0.4i
H3	66.7±0.4a	5.8±0.5bc	46.8±0.4ab	0.12±0.01de	47.6±0.3ab	82.9±0.6ef
H3+3d	65.3±0.3ab	8.1±0.4a	46.9±0.4ab	0.17±0.01ab	47.7±0.4ab	80.2±0.5hi
H4	62.4±0.8de	4.7±0.4e	43.0±0.5d	0.11±0.01e	43.4±0.5d	83.7±0.5e
H4+3d	54.7±1.0g	6.6±0.4ab	39.7±0.6e	0.15±0.01bc	40.2±0.6e	81.4±0.5gh

5.4.2 Phytochemical composition

Phenolic content of 'Karantoki' ranged from 40.4±1.1 to 48.7±2.8 and from 11.8±0.8 to 27.3±0.5 GAE mg 100^{-1} FW for free and bound phenolics, respectively. The respective values for 'Morphitiki' cultivar were in the range 46.0±2.0 to 53.9±0.2 and 19.1±4.5 to 31.5±2.7 GAE mg 100^{-1} FW for free and bound phenolics compounds. Free phenolics (FP) contributed by 63.6-78.4 % of total phenolic content for 'Karantoki' and

[17] *Results are the means ± standard error (n=30). Different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).*

62.5-71.8 % for ‘Morphitiki’ **(Figure 5.1)**. Early harvest for both cultivars was linked with higher phenolic contents, both in their free and bound form. Gruz et al., (2011) mentioned that the decrease of bound phenolics during the progress of fruit maturation is due to their alteration to forms that are no longer detectable by spectrophotometry. They have also indicated that the reduction in free and bound phenolics is related to the decrease of primary metabolism substrates that leads to a decline of the phenolic compound biosynthesis.

Contrarily, Kevers et al. (2014) postulated that dark cherries and red raspberries presented higher phenolic and antioxidant capacity at their fully ripe stage in comparison with other dark fruits that had ambiguous or no differences at the harvesting period, indicating also that the antioxidant capacity varied according to harvest time, maturity stage and cultivation practises. Ding et al. (2001) reported that at the ripe stage of seven Japanese loquat cultivars, the total phenolic content was highly variable, ranging from 81.8 up to 173.8 mg 100 g^{-1} FW. Chlorogenic acid was the predominant compound, accounting for up to half of the total phenolics.

‘Morphitiki’ registered higher total phenolic content, mainly due to the higher bound phenolics content (**Figure 5.2**). In an earlier study of our group, ‘Morphitiki’ also registered higher free phenolic content than ‘Karantoki’ (Goulas et al., 2014). Acosta-Estrada et al. (2014) indicated that the percentage of bound phenolics over the total phenolic content varies significantly, i.e. 6.5 % in apple, 33.1 % in banana, 24.3 % in orange and 20.7 % in medlar fruits. Comparing the bound phenolic contend of 11 common fleshy fruits, Sun et al. (2002) reported on average a 24 % of the total content derive from the bound form. Based on their contribution in total phenolic fraction, the determination of bound phenolics needs to be taken into account.

In the current study, no evident differences between the harvest date and the shelf life period for both cultivars regarding the free phenolic content were monitored. The bound phenolics presented the highest values at H1 (25.8±1.6 GAE mg 100^{-1} FW) and H1+3 (27.3±0.5 GAE mg 100^{-1} FW) for ‘Karantoki’ cultivar. Similarly, ‘Morphitiki’ registered higher FP values at H2 and H3 (51.7±0.4 to 53.9±0.2) as well as BP values at the same harvesting days (24±0.8 to 31.5±2.7); higher values for both free and bound phenolics were registered at H2+3, 53.9 and 31.5 GAE mg 100^{-1} FW, respectively (**Figure 5.1**).

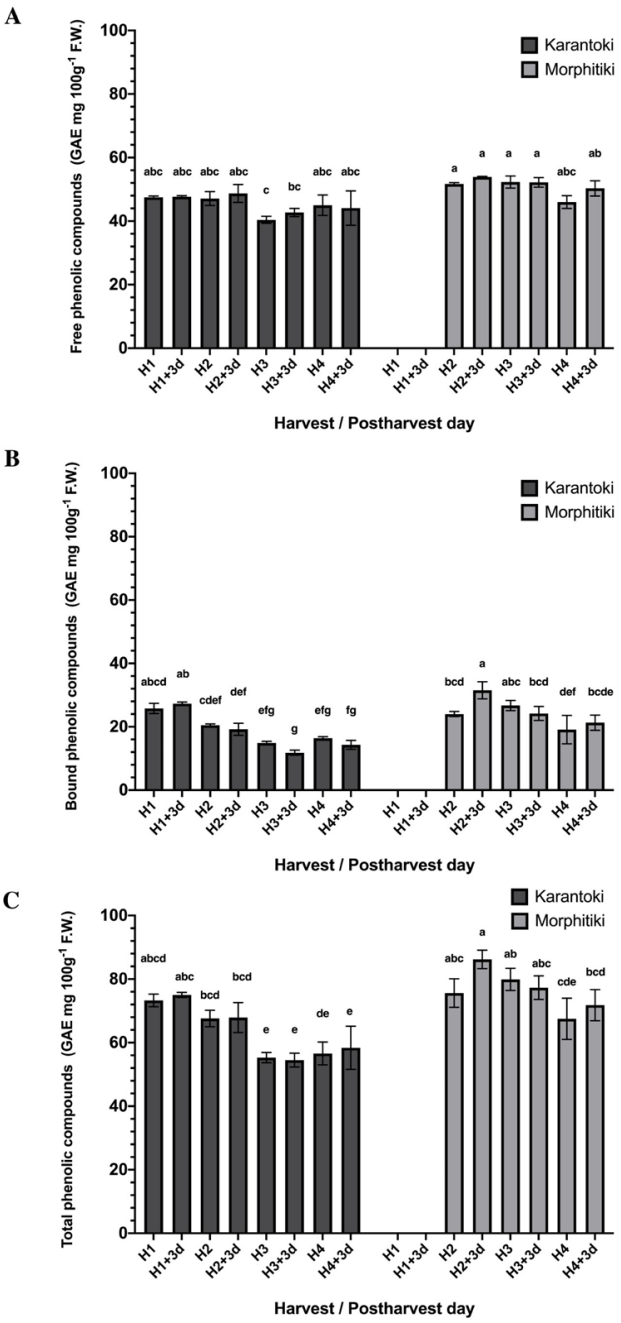

Figure 5.1: Free (A), bound (B) and total phenolic content (C) of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days.[18]

[18] *Harvest dates are indicated in* **Table 5.1**. *Results are the mean ± standard error (n=3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).*

Overall, bound phenolics registered a pronounced decrease at advanced harvested ‘Karantoki’ fruits whilst bound phenolics for ‘Morphitiki’ fruits had negligible changes during the harvesting period. The non-significant changes of bound phenolics in ‘Morphitiki’ fruits can be partially explained by the slight or no changes at SSC from different harvesting dates (**Table 5.2).** The release of phenolic compounds is a possible explanation for this trend since the bound phenolics are covalently bound to sugar moieties or cell wall structural components with the progress of ripening. It should be also taken into account that the Folin–Ciocalteu assay does not detect all phenolic groups found in the extracts and it cannot monitor the release of bound phenolics as free (Stalikas, 2007).

5.4.3 Antioxidant capacity

Antioxidant capacity was determined with the employment of DPPH and FRAP assays and results for both free and bound phenolics are presented. The DPPH and FRAP values for the free phenolics showed higher content in comparison with the bound phenolics due to their abundance in the free fraction. More specific the free DPPH values (μmol Trolox 100^{-1} FW) ranged from 184.6±10.7 to 257.9±6.9 and from 243.2±3.4 to 277.7±8.9, whereas the bound DPPH values ranged from 71.3±4.9 to 179±3.6 and from 140.2±10.9 to 180.7±6.6 for ‘Karantoki’ and ‘Morphitiki’, respectively (**Figure 5.2**). Similarly, free FRAP values (μmol Trolox 100 g^{-1} FW) ranged from 178.2±17.1 to 226.1±4.2 for cv. ‘Karantoki’ and 123.7±2.3 to 148.7±6.5 for cv. ‘Morphitiki’. The bound FRAP values ranged from 52.8±13.1 to 137.3±2.7 and from 64.5±6.7 to 144.1±12.1 for cv. ‘Karantoki’ and cv. ‘Morphitiki’ respectively (**Figure 5.3**). Goulas et al. (2014) postulated also higher DPPH and FRAP antioxidant capacity for the ‘Morphitiki’ cultivar in comparison with ‘Karantoki’, without however taking into account the bound form.

The highest values for both cultivars for DPPH (free and bound) were registered during the first harvest date and shelf life for cv. ‘Karantoki’, H2, H3 and shelf life for cv. ‘Morphitiki’. Bound FRAP had higher values for the H1 and H2 for the ‘Karantoki’ cultivar and their respective shelf life periods. Similarly, ‘Morphitiki’ had higher bound FRAP content at H2+3. For both cultivars, these values went descending after advanced harvesting days and the subsequent shelf life periods (**Figure 5.2; Figure 5.3**).

High positive correlation between the phenolic content and the antioxidant capacity of loquat fruit is manifested in accordance with previous studies both for loquat fruits (Xu and Chen, 2011; Goulas et al., 2014; Zhang et al., 2015) and other fruit tissues (Gil et al., 2002; Sun et al., 2002; Kevers et al., 2014).

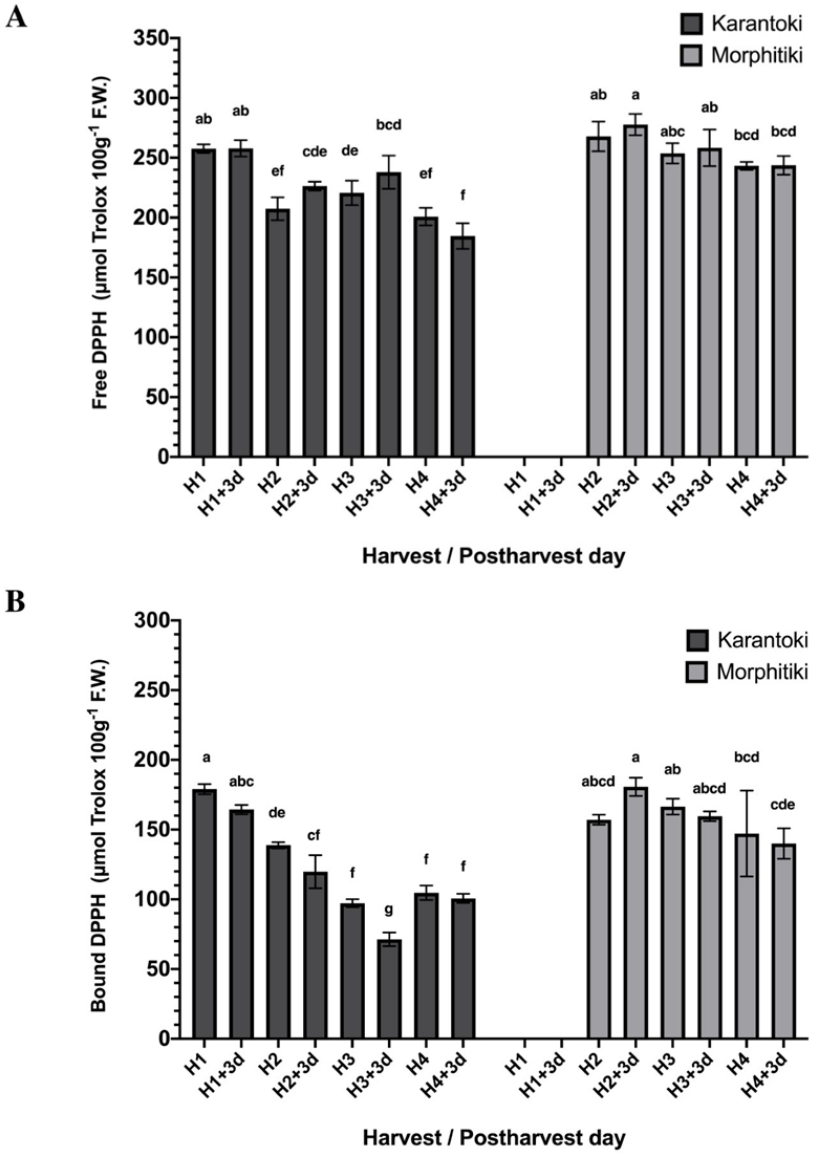

Figure 5.2: DPPH free (A) and bound (B) antioxidant capacity of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days.[19]

[19] *Results are the mean ± standard error (n=3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).*

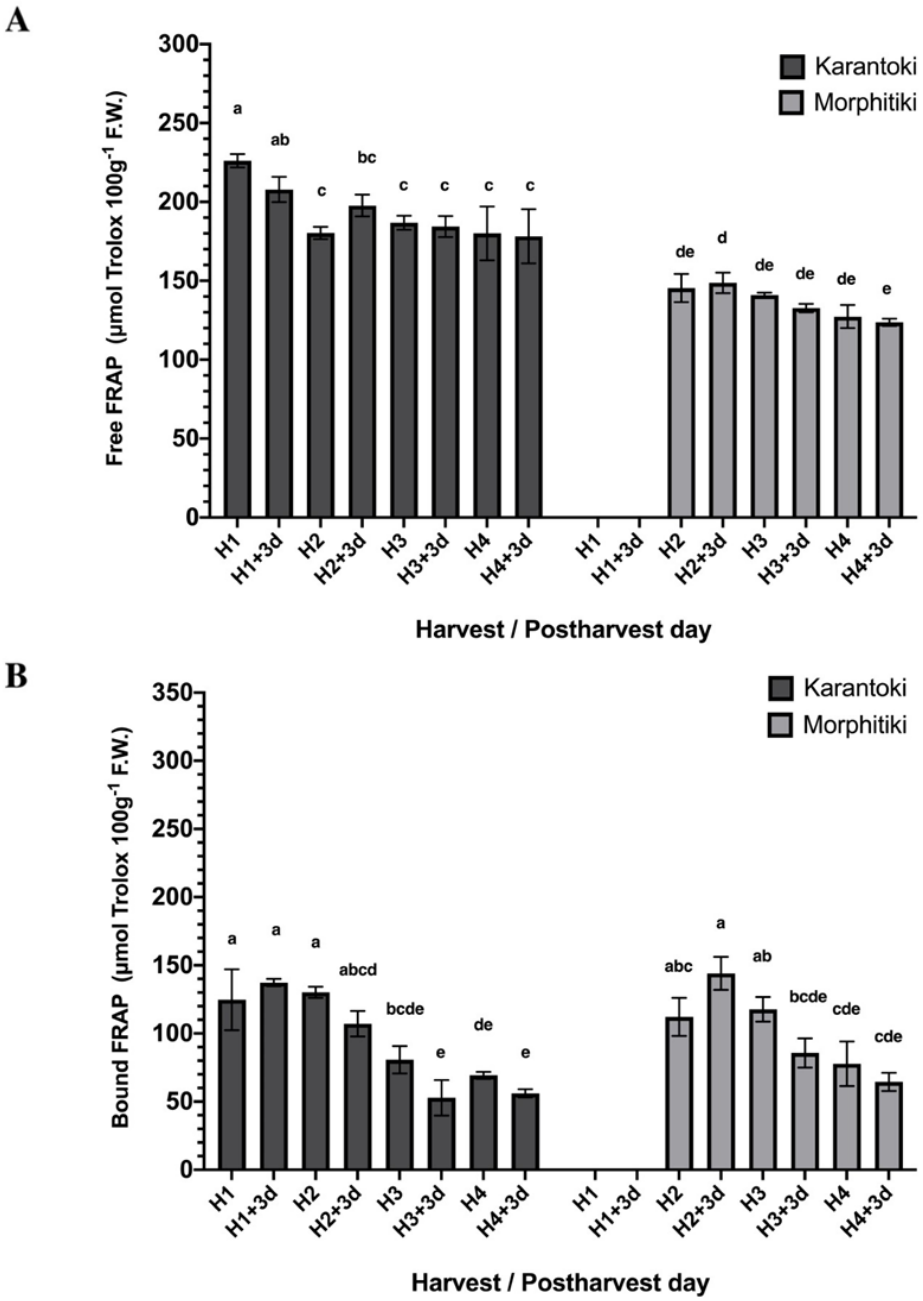

Figure 5.3: FRAP free (A) and bound (B) antioxidant capacity of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days.[20]

[20] ******Results are the mean ± standard error (n=3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).*

The correlation coefficients (*r*) between phenolic content and antioxidant capacity were additionally calculated to dissect the main loquat components that are responsible for antioxidant capacity. A strong correlation was found between bound phenolics and DPPH as well as between bound phenolics and FRAP capacity (0.954 and 0.827), highlighting that significant amounts of antioxidant phenolics were revealed after hydrolysis. On the contrary, the correlation coefficients between total phenolics and antioxidant capacity in free fraction was lower (0.682 and -0.495). Such results can be linked with the potential presence of other groups of antioxidant compounds, such as carotenoids and ascorbic acid.

5.5 Conclusions

There are variations between the qualitative characteristics of the loquat fruit in relation to the harvest date and the cultivar considered. Early harvested 'Karantoki' fruits were characterized by less appreciable qualitative properties, evident by low a*/b* ratio, decreased SSC and elevated TA values. Early harvests and the corresponding shelf life periods for both cultivars were linked with higher phytochemical content compared to late harvests, evident by higher antioxidant capacity, free and bound phenolic content. A highly positive correlation between the bound phenolic content and the antioxidant capacity of loquat fruit was monitored. 'Morphitiki' was characterized by higher values for all harvest dates; highlighting the genotype significance.

6 The efficacy of preharvest foliar spray applications on yield efficiency, incidence of physiological disorders and shelf life performance of loquat fruit

6.1 Abstract

The effect of preharvest foliar spray applications at fruit color breaker stage with acetylsalicylic acid (synthetic salicylate), spermidine (polyamine), their combination and a commercial calcium supplement on loquat (Eriobotrya japonica Lindl cv. 'Karantoki') fruit quality at successive harvests and subsequently during maintenance at room temperature (shelf-life) for 3 and 6 days respectively, were investigated. Yield efficiency, qualitative attributes, textural properties and the incidence of physiological disorders, namely purple spot and fruit cracking, were determined. Acetylsalicylic acid is recommended as an efficient treatment that needs to be further explored towards production of loquat fruit with enhanced properties. Furthermore, the calcium supplement appeared to present some promising results. However, spermidine application alone or in combination with acetylsalicylic acid did not show any beneficial and/or synergistic effect on loquat fruit quality parameters to justify its application. Foliar applications did not affect fruit growth and yield efficiency. The current study shed some light on the potential use of foliar spraying with acetylsalicylic acid, alone or potentially in combination with a calcium supplement, towards enhancement of fruit quality properties of an added value product as loquat. To this aim, further studies to test their efficacy under different environmental conditions that may accelerate the incidence of physiological disorders and/or multiple applications over the growing season need to be implemented.

Keywords: *Eriobotrya japonica*, harvest, purple spot, preharvest treatments, preharvest disorder, polyamine, acetylsalicylic acid, spermidine

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6.2 Introduction

Loquat (*Eriobotrya japonica* Lindl) is a subtropical evergreen tree, the fruit of which is highly appreciated for its light refreshing taste, being recommended as an alternative commodity to mainstream fruit crops (Gisbert et al., 2009; Goulas et al., 2014; Hadjipieri et al., 2020a). Under Mediterranean conditions, the tree blooms in late autumn-early winter and the fruits are being harvested during the early spring period (Badenes et al., 2013), when there is a lack of availability of other fresh fruits (Hadjipieri et al., 2017). On the other side, loquat is a highly perishable commodity, suffering by physiological disorders both on tree (purple spot, fruit cracking) and after cold storage, that are considered main limiting factors on fruit marketability (Hadjipieri et al., 2020b; Munera et al., 2021). It is additionally characterised by scalar ripening and the need for successive harvests and high overall production costs. The efficacy of preharvest foliar application of chemical agents, such as plant growth regulators, towards increment of yield efficiency and enhancement of qualitative properties has received accumulating interest over the recent years in an array of fruit crops, yet not in loquat fruit.

Polyamines are growth regulators that have been widely implicated to combat both abiotic and biotic stresses (Fortes et al., 2019). Exogenous application of polyamines have been reported to delay ripening-related softening, reduce mechanical damage and chilling injury (CI) sensitivity and therefore increase shelf life in both climacteric and non-climacteric fruits (Davarynejad et al., 2015). Spermidine (Spd) together with putrescine and spermine are the three most common polyamines, ubiquitously found in all living organisms. The former (Spd) is known to be synthesized from putrescine through the arginine pathway by spermidine synthase with the aminopropyl moiety decarboxylated *S*-adenosylmethionine (Liu et al., 2006; Sharma et al., 2017). Spermidine is involved in an array of developmental processes in higher plants, including embryogenesis, cell division, senescence and biotic and abiotic stress such as nutrient deficiency, hyperosmosis, temperature stresses, wounding and hypoxia (Pang et al., 2007; Davarynejad et al., 2015; Sharma et al., 2017; Sequera-Mutiozabal et al., 2017). Overall, polyamines are associated with fruit set, ripening, and regulation of fruit quality-related traits and their application has been additionally linked with an inhibitory effect towards postharvest decay (Fortes et al., 2019).

Acetylsalicylic acid (ASA), a derivative of salicylic acid, is considered as a plant growth regulator with important roles in a wide range of physiological processes, such as flowering, seedling germination, fruit development, organogenesis and biotic and abiotic stress responses (Giménez et al., 2014; Martínez-Esplá et al., 2017; Koo et al., 2020; Antoniou et al., 2020). ASA is converted to salicylic acid spontaneously when applied exogenously (Sayyari et al., 2011; Giménez et al., 2014) and its postharvest application on loquat fruit has been reported to alleviate CI symptoms after cold storage (Cai et al., 2006).

Calcium application in the form of preharvest spray is a typical strategy that is being applied in apple orchards as a means to combat bitter pit disorder that has been attributed to calcium deficiency (Torres et al., 2017). Preharvest calcium application is also a common strategy employed for kiwifruit (Gerasopoulos and Drogoudi, 2005). However, among the external factors that regulate fruit Ca accumulation are the meteorological variables that affect fruit transpiration (Montanaro et al., 2015). Notably, a comparative study among different fruit crops showed that fruit Ca uptake rate was the highest in pome fruits such as apple, pear and loquat (Song et al., 2018). Calcium treatment is for several loquat orchards a standard strategy and empirical data have shown to control the incidence of purple spot physiological disorder, particularly in growing seasons that induce intense symptoms of this disorder. Only recently, exogenous calcium applications has been also reported to effectively enhance the chilling tolerance of loquat fruit (Li et al., 2020), without however providing any evidence regarding its efficacy on preharvest physiological disorders.

Loquat is a largely unexplored crop and to our knowledge the efficacy of such chemical compounds in the form of preharvest foliar spay applications have not been dissected in this crop. Therefore, the aim of the current study was to explore the potential beneficial effect of an array of preharvest applications on loquat fruit qualitative attributes.

6.3 Materials and Methods

6.3.1 Fruit material

The experiment was performed in cv. 'Karantoki', the most widely grown cultivar in Cyprus. In particular, fifteen 19-year-old loquat trees of similar vigor and canopy uniformity were used according to cultivation techniques described in §2.1.

6.3.2 Methodology

Foliar application of 15% (w/w) Ca-chloride hydrate (CaCl2* H_2O) (Meda Ca, Solfotecnica Italiana S.p.a, Italy), 2 mM spermidine (Spd), 2 mM acetylsalicylic acid (ASA) and a combination of 2 mM Spd and 2 mM ASA were applied on day 54 (23rd of February, 2019), corresponding to 9 weeks after full bloom in a complete randomized design. Spd and ASA were purchased from Sigma-Aldrich (St Louis, MO, USA). All chemical reagents were dissolved to water as per manufacturer instructions. Foliar applications were made using freshly prepared solutions and an adhesive substance (Nu-Film-P, Pinene 96%, Miller Chemical and Fertilizer, LLC, USA) was also added. Sprayings were made with an air assisted mist sprayer (Cifarelli series L3, Vochera, Italy; **Figure 6.1**). Control trees received spraying with water. Three single tree replicates for each treatment were chosen in completely randomized blocks in the middle of the orchard.

6.3.3 Fruit growth, yield efficiency and fruit quality classification

Fruit growth was determined by measurements of length (L) and width (W) dimensions in 10 representative fruits per treatment applied. Physical dimensions of fruit were determined with the employment of an electronic caliber (IS11112, Insize; **Figure 6.2**) in a 7-day interval. The L/W ratio was calculated, being a measure of sphericity. Fruit dimension recordings took place until few days prior to first harvesting day.

Fruit were harvested, based on fruit size and external color, at day 95 (H1), 109 (H2) and 126 (H3) (5th April, 19th April and 6th May). The fruit of each harvest were segregated into commercial categories defined according to the Cypriot fruit market standards, namely extra (weight over 40 g), A (weight between 30-40 g) and B (weigh

less than 30 g). All these categories included fruit without blemishes and/or any visual defect. Fruit with incidence purple spot (PS) symptoms that accounted for more than 10% of its surface was assigned in the category ‘purple spot’ (**Figure 6.3A**) and fruit with cracking symptoms was categorised as ‘cracking’ (CR) (**Figure 6.3B**). Detailed description of the PS incidence is presented in §2.3. The percentage (%) yield, in each fruit quality category, and the yield efficiency, as the ratio of total yield and trunk cross section area, were calculated. Trunk cross section area was determined by the mathematical equation $\pi*(P/2)^2$ where P stands for tree perimeter.

Figure 6.1: Tree spraying with an air assisted mist sprayer.

Figure 6.2: Successive on-tree developmental stages cv 'Karantoki' under the different treatments [Control, 15% w/w Meda Ca, 2 mM spermidine (Spd), 2 mM acetylsalicylic acid (ASA) and a combination of 2 mM Spd and 2 mM ASA (ASA + Spd)]. [21]

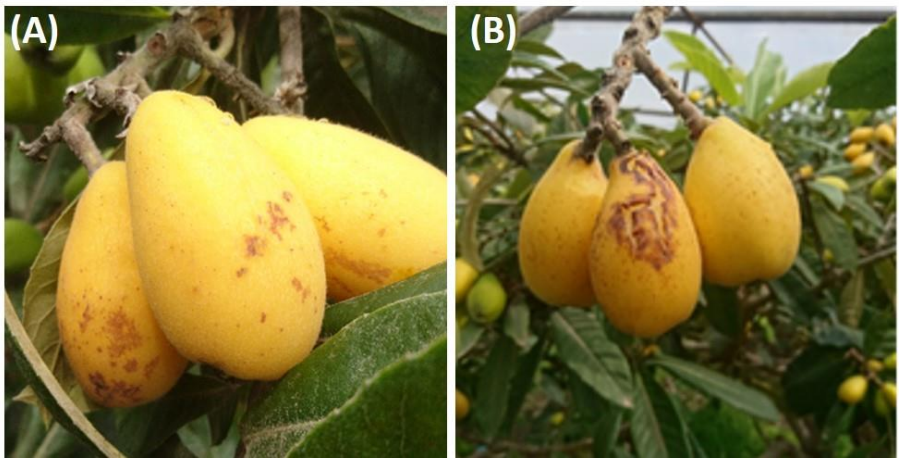

Figure 6.3: Defective loquat fruits due to the incidence of the physiological disorder purple spot (A) and to fruit cracking (B).

[21] 'WAFB' stands for weeks after full bloom.

A homogeneous lot of 90 representative fruits, free of any visual defects, (divided in three 30-fruit sublots) were selected per each of the three harvesting days. Such fruits were analyzed at harvest (H) and after additional maintenance at room temperature for 3 (H+3) and 6 days (H+6), respectively. Weight loss (%), color parameters (L*, a*, b*, Chroma and hue angle), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) were determined as described in §2.2.1; 2.2.3; 2.2.2; 2.2.4 and 2.2.6 respectively. Ripening index (RI) was calculated as the SSC/TA ratio.

6.3.4 Statistical analysis

The fruit growth measurements (L, W and L/W) were subjected to repeated measure analysis of variance, having the initial values of each fruit as a covariate. A multi-factor ANOVA was performed for yield measurements (chemical and harvest date as treatments) and fruit quality measurements (chemical, harvest date and shelf-life as treatments), based upon the replicate tree or the technical fruit replicates. Treatment means were separated using a Duncan multiple range test or least significant difference (LSD) comparisons where ANOVA F-tests were significant, using a *P*-value of 0.05. Percentage data were arcsine transformed, while ratio data were log-transformed prior to analysis. The statistical package SPSS v22.0 (SPSS, Inc., Chicago, IL, USA) was used. Graph illustrations were performed using Prism 8 (GraphPad, Lo Jolla, CA).

6.4 Results

6.4.1 Fruit growth, yield and quality categories at harvest

Loquat fruit followed a typical sigmoid curve, in accordance with other studies (Su et al., 2017; Hadjipieri et al., 2020b) (**Figure 6.4**); however, no significant effect of any chemical treatment on fruit growth measurements or interaction with time was monitored **(data not shown)**.

Fruit yield (taking into account all harvest dates and chemical treatments applied) in different quality categories, based on fruit size and presence of blemishes, was highest in ‘Extra’ (70.8%), followed by Category ‘A’ (22.9%); a minor proportion

of fruits (3.1%) with weight below 30 g (Category 'B') was registered. The physiological disorders CR and PS were detected in trace amounts, registering values of 2.0% and 1.2% of total fruits, respectively (**Table 6.1; Supplementary Table S7**). However, yield efficiency was not affected by the different chemical treatments applied **(data not shown).**

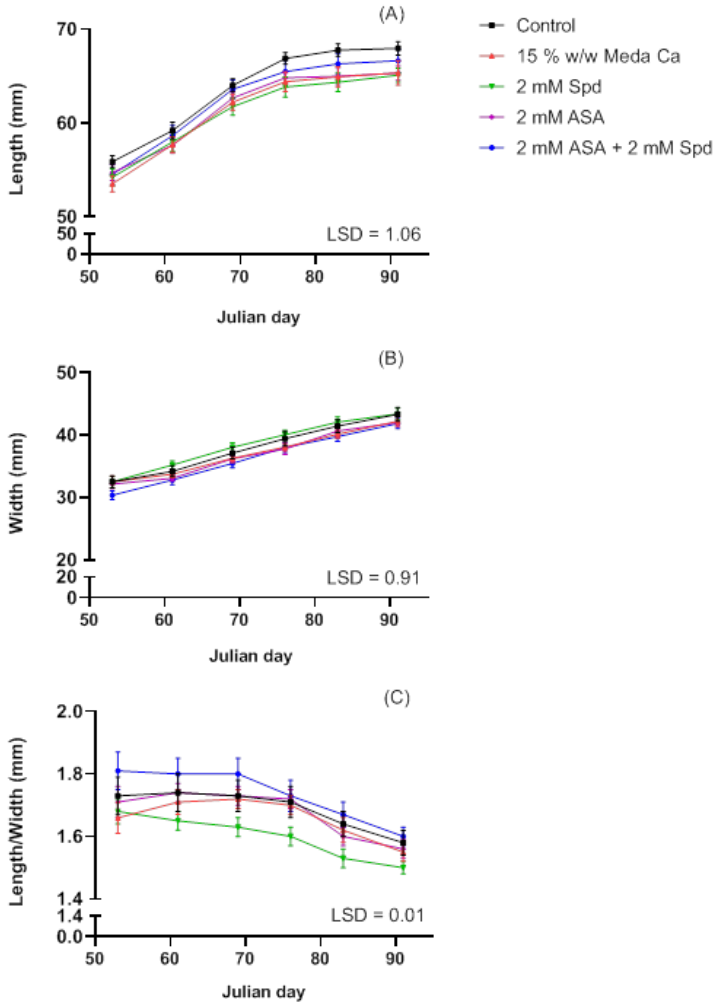

Figure 6.4: Length (A), width (B) and length/width (C) of 'Karantoki' fruits subjected to 5 different preharvest treatments at 6 preharvest dates (23rd February, 2nd March, 10th March, 17th March, 24th March and 1st April).

The chemical treatments did not affect the yield proportion in different quality categories, but there was a significant interaction between chemicals and fruit quality categories, showing that the ASA-treated trees had practically non-detectable incidence

of purple spot. Although control trees also showed low PS incidence (2.4%), this was statistically higher compared to ASA-treated trees (**Table 6.1; Figure 6.5**).

Table 6.1: Effects of chemical treatments and harvest times on % fruit yield per each fruit category.

	% Extra	% A	%B	% PS	% CR
Chemicals					
Control	67.4 ±4.3 ab	24.8±4.6a	3.3 ±0.8a	2.4 ±1.6 a	2.1 ±0.3a
2 w/w Meda Ca	78.4 ±1.8 a	17.1 ±2.1a	1.8 ±0.2a	1.3 ±0.3 ab	1.4 ±0.5a
2 mM Spd	73.4 ±3.5 ab	20.9 ±3.0a	2.1 ±0.5a	1.8 ±0.5 ab	2.2 ±0.7a
2 mM ASA	69.6 ±2.3 ab	23.9 ±1.9a	3.9 ±0.7a	0.2 ±0.2 b	2.7 ±0.2a
2 mM ASA + 2 mM Spd	65.1 ±1.8 b	27.8 ±1.0a	4.9 ±0.4a	0.7 ±0.3 ab	1.5 ±0.6a
Harvest date					
Harvest 1	71.9± 5.6 a	21.1±4.9 b	1.3±0.6 c	0.9±0.6a	4.7±1.1 a
Harvest 2	75.5±5.0 a	19.8±4.2 b	2.7±1.2 b	1.2±0.6a	0.8±0.2 b
Harvest 3	59.5±2.8 b	31.9±3.0 a	6.6±1.3 a	1.6±1.1a	0.5±0.3 c

Means with different letters indicate statistical differences ($p<0.05$) across each fruit quality category in chemical and harvest date treatments separately.

Late harvested fruit (H3) registered a lower proportion of premium fruits (category 'Extra'), suggesting a significant interaction between fruit quality classification and harvest date (**Table 6.1**; **Table 6.2**). CR symptoms were higher at initial harvest (4.7%), followed by substantially lower incidence over the more advanced harvesting days were only trace amounts were monitored.

Table 6.2: *P* values from the multi-factor ANOVA performed on the effects of chemicals, harvest date, fruit quality categories and their interactions, on the percentage (%) of total yield, and the repeated measure analysis performed for the fruit growth measurements (initial measurements were used as a covariate).

	Yield (%)
Chemical	ns
Harvest	ns
Categories	<0.001
Chemical * Harvest	ns
Chemical * Categories	<0.001
Harvest * Categories	<0.001
Chemical * Harvest * Categories	ns

ns, non-significant

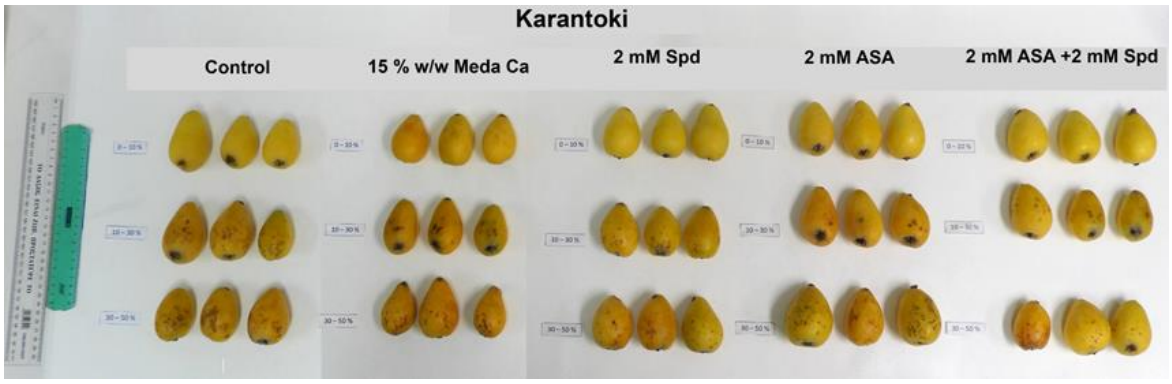

Figure 6.5: Severity Incidence (SI) categorization for purple spot appearance on cv 'Karantoki' under five different preharvest treatments at fully-ripe stage.

Table 6.3: Treatment means and P values for the effects of chemicals, harvest and shelf life and their interactions on flesh firmness(FF), weight loss (%), soluble solids content (SSC), titratable acidity (TA) ripening index (RI) and color parameters of 'Karantoki' fruits.[22]

	WL %	FF	L*	a*	b*	Chroma	Hue Angle	a*/b*	SSC	TA	RI
Chemicals											
Control	9.3 a	1.045 a	63.8 a	3.3 a	48.6 a	48.7 a	86.1 c	0.067 a	12.9 a	2.7 b	58.9 a
15 % w/w Meda Ca	8.2 d	1.045 a	61.5 c	2.8 bc	46.1 c	46.2 c	86.3 b	0.061 ab	12.2 b	3.3 a	43.9 c
2 mM Spd	8.9 b	1.009 b	61.5 c	2.6 c	45.4 d	45.5 d	86.5 b	0.056 b	12.3 b	2.6 b	51.1 ab
2 mM ASA	8.5 c	1.023 b	61.4 c	2.0 d	45.2 d	45.3 d	87.3 a	0.044 c	12.0 b	2.7 b	50.5 b
2 mM ASA + 2 mM Spd	9.3 a	1.048 a	62.3 b	3.0 ab	47.6 b	47.8 b	86.2 c	0.064 a	11.4 c	2.5 b	52.6 ab
Harvest date											
Harvest 1	8.0 c	1.074 a	64.7 a	1.8 c	49.7 a	49.7 a	87.6 a	0.036 c	11.3 b	3.3 a	28.4 c
Harvest 2	10.5 a	1.036 b	59.0 c	2.5 b	46.0 b	46.2 b	86.7 b	0.055 b	11.2 b	2.9 b	43.8 b
Harvest 3	8.1 b	0.992 c	62.6 b	3.9 a	44.1 c	44.2 c	85.2 c	0.084 a	14.1 a	2.2 c	71.7 a
Shelf life											
0+		1.027 b	63.6 a	1.6 c	44.9 c	45.0 c	87.6 a	0.035 c	11.6 c	3.7 a	34.1 c
3+	6.1 b	1.045 a	62.0 b	2.8 b	46.2 b	46.3 b	86.5 b	0.060 b	12.2 b	2.7 b	49.3 b
6+	11.7 a	1.030 b	60.7 c	3.9 a	48.6 a	48.8 a	85.4 c	0.080 a	12.6 a	2.0 c	69.9 a
P values											
Chemical	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Harvest	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Shelf life	<0.001	0.034	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Chemical * Harvest	<0.001	ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.019	0.036
Chemical * Shelf life	ns	ns	ns	ns	ns	ns	ns	ns	0.033	ns	ns
Harvest * Shelf life	<0.001	<0.001	<0.001	0.027	<0.001	0.004	<0.001	ns	<0.001	ns	0.008
Chemical * Harvest * Shelf life	ns	0.011	0.036	ns	0.036	ns	0.035	ns	<0.001	ns	ns

[22] Mean values are shown on **Figure 6.6; Figure 6.7; Figure 6.8.**

6.4.2 Qualitative attributes

The calcium treatment exhibited the lower weight loss compared with control, especially in H1 and H2, followed by ASA and Spd-treated trees, whilst ASA + Spd had no effect on weight loss (**Figure 6.6A**). Fruit weight loss was above the threshold of 10% after 6 days (11.7 %), an indicator of fruit shrivelling and deterioration.

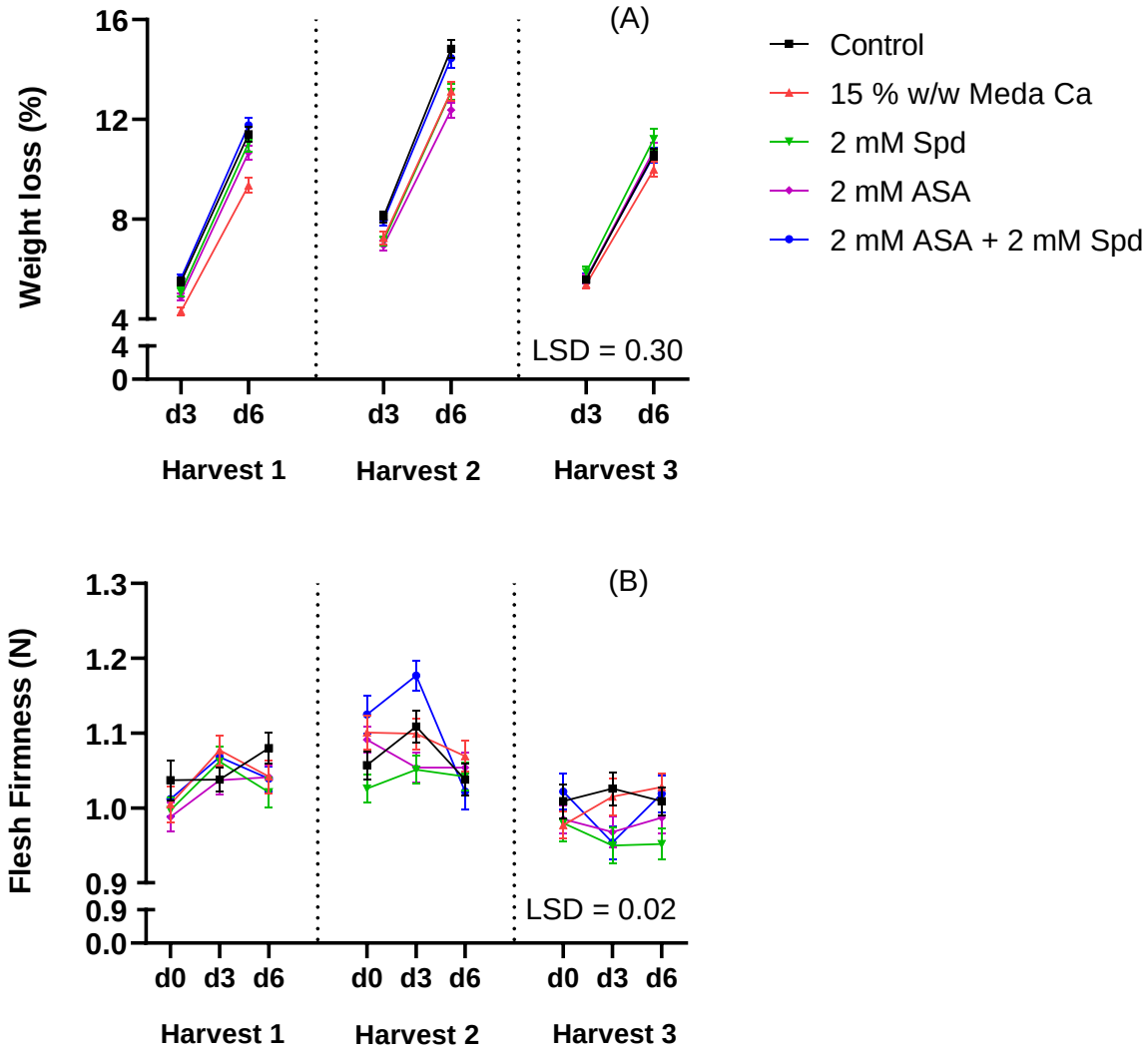

Figure 6.6: Flesh firmness (A) and Weight loss (B) of 'Karantoki' fruits subjected to 5 different preharvest treatments at harvest (H1-H3) and after additional maintenance at room temperature for 3 and 6 days.

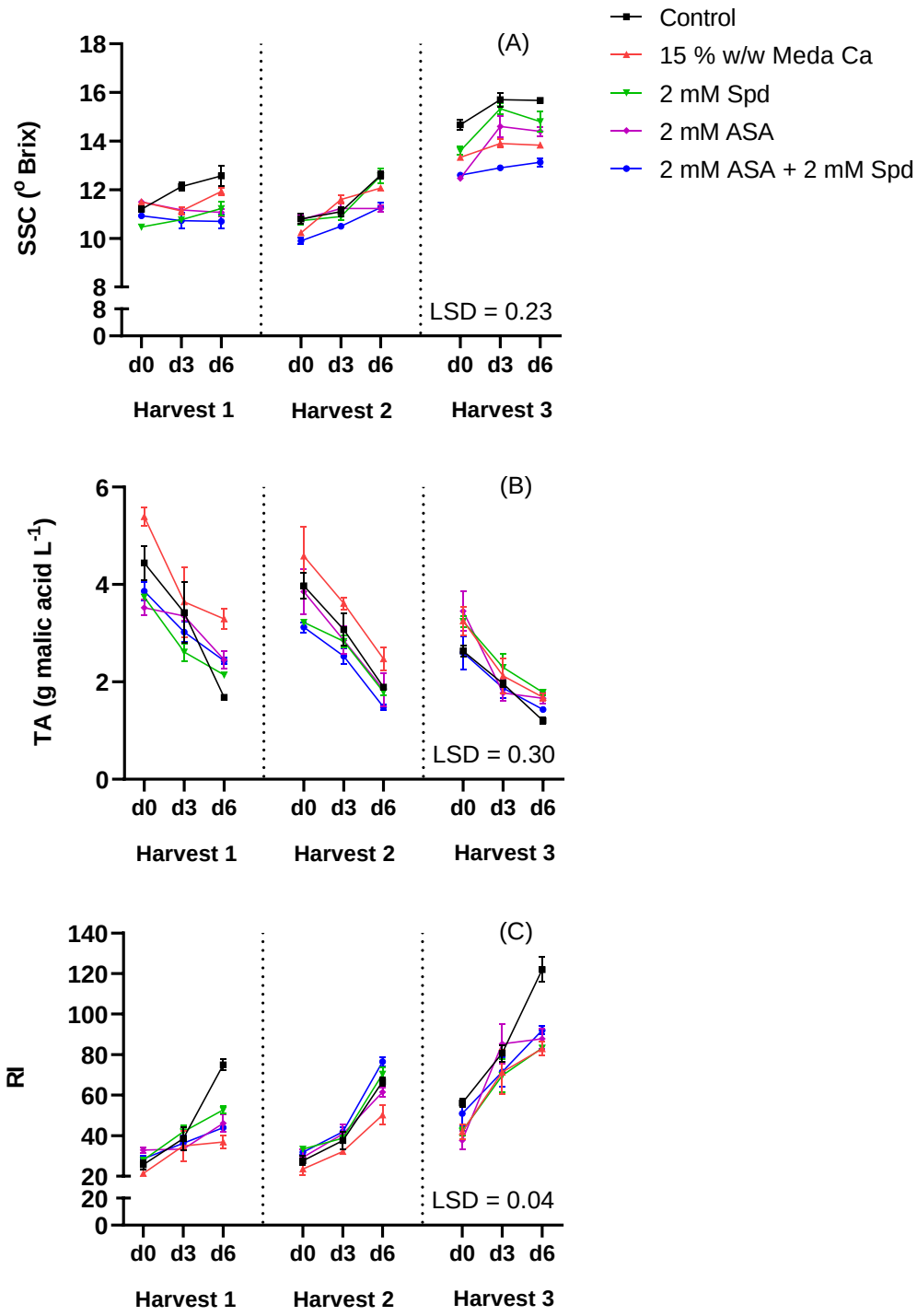

Figure 6.7: Soluble solids content (SSC) (A), titratable acidity (TA) (B) and ripening index (RI) (C) values for 'Karantoki' fruit, subjected to 5 different preharvest treatments at harvest (H1-H3) and after additional maintenance at room temperature for 3 and 6 days

Generally, all chemical treatments, and to a greater extent the combination of ASA + Spd, showed reduced SSC values compared with control that might imply a redox reaction. The effect was more pronounced in the more advanced harvesting times (**Figure 6.7A; Table 6.3**). TA contents presented the highest values on fruits treated with the calcium supplement (**Figure 6.7B**). TA contents was highest in H1, followed by H2 and H3, with a reduction of 12.0 and 32.6 % respectively, whereas shelf life gradually decreased the TA value accordingly from 25.4% at 3 days shelf life up to 46.6% after 6 days of shelf life. Calcium-treated fruits were generally characterized by lower RI for all harvesting times, while a similar effect was evident by the rest chemical treatments only during H3. As expected, there was a progressive increase of RI during the successive harvests and shelf life periods (**Figure 6.7C; Table 6.3).**L* values were generally lower in all chemical treatments, compared with control, in H2 and H3 (**Figure 6.8A; Table 6.3**). A reduction in L* values was also noted in H2 and H3, compared with H1, and additionally after 3 and 6 days of shelf life. All chemical treatments exhibited lower a* values compared with control at H1, whereas at H2 they were lower only in ASA, and in H3 only in ASA and Spd (**Figure 6.8B; Table 6.3**). The a* values increased by 36.6 % at H2 and by 54.4% at H3, compared with H1. Similarly, a* increased by 43.4% at H+3 and 59.4% at H+6, compared with the harvesting day. Concomitant with the abovementioned results are the b* values which followed a similar trend. ASA and Spd exhibited the lowest values in all three harvests. Calcium-treated fruit registered the lower values in the late harvests (H2 and H3) (**Figure 6.8C; Table 6.3**).

The a*/b* ratio exhibited lower values for all treatments at H1 and the two shelf life periods applied (+3 and +6) in correlation with control and thereafter ASA was noted for the reduced values in the next two harvests and the corresponding shelf life periods (**Figure 6.8D; Table 6.3**). Lower values were also noted for Spd at H3 and after 3- and 6-days maintenance at room temperature. Statistically significant differences were generally registered for the a*/b* ratio treated with Spd and ASA as the treatments lead to an increase by 15.1% and 34.0%, respectively. Chroma values were lower in all treated fruit compared with control for all harvest dates (**Figure 6.8E; Table 6.3**).

Chroma values were lower in H2 and H3, compared with H1, and increased during shelf-life ripening. ASA and Spd-treated fruit were generally characterized by higher hue angle values, particularly during the initial and the last harvest date (**Figure 6.8F; Table 6.3**). Hue angle was lower for all treatments at H3, being further decreased during maintenance at room temperature.

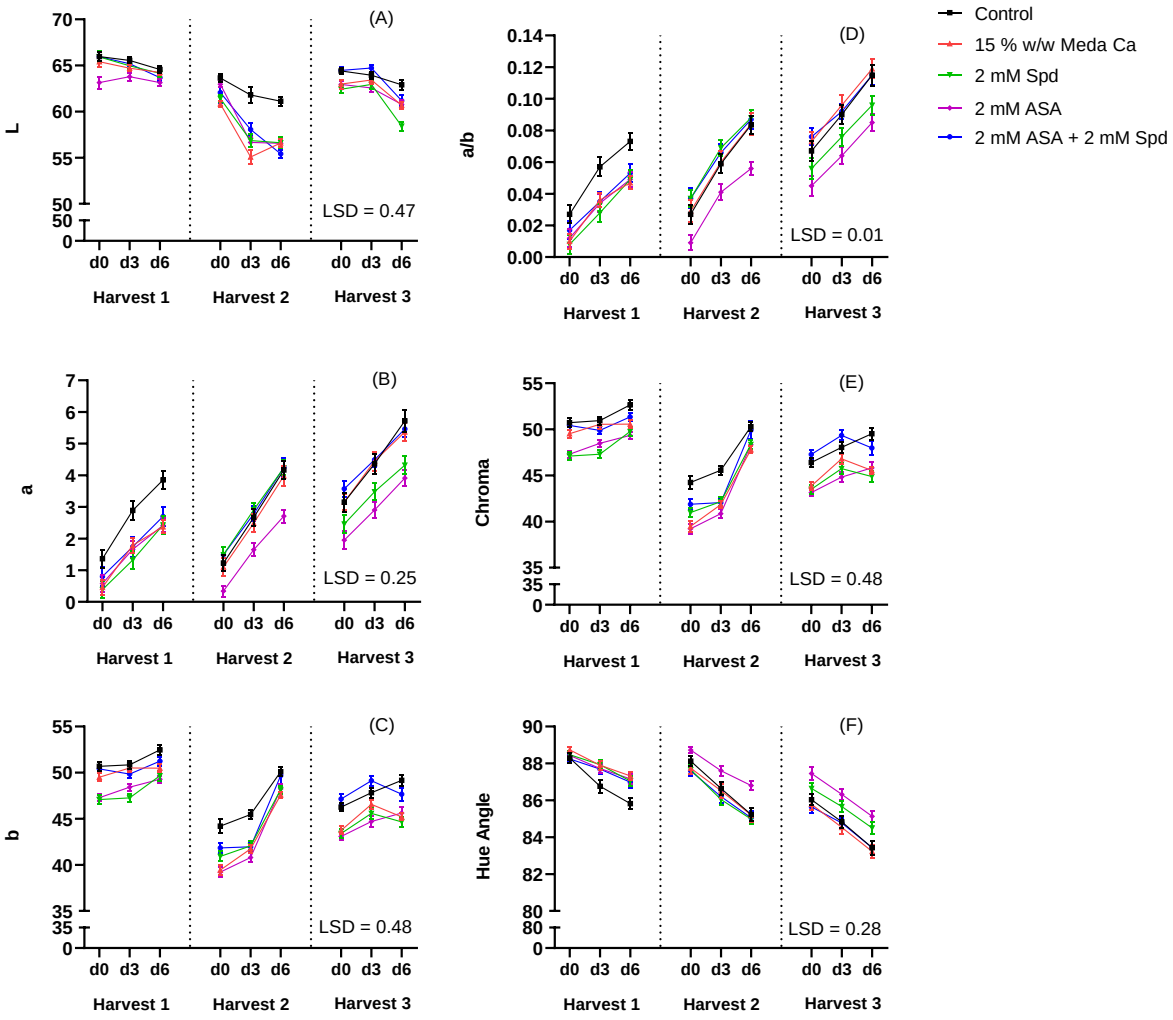

Figure 6.8: Color parameters [L* (A), a* (B), b* (C), a*/b* (D), chroma (E) and hue angle (F)] of 'Karantoki' fruits subjected to 5 different preharvest treatments at harvest (H1-H3) and after additional maintenance at room temperature for 3 and 6 days.

6.5 Discussion

6.5.1 Effects of chemical treatments

The concentrations of 2 mM ASA (strawberry, Babalar et al., 2007; sweet cherry; Giménez et al., 2014; pineapple; Lu et al., 2011; pomegranate; Sayyari et al., 2009) and 2 mM Spd (kiwifruit; Jhalegar et al., 2012) were chosen based on previous studies where a beneficial effect of such concentrations has been reported.

ASA-treated trees had the lowest incidence of purple spot and fruit quality analyses showed that it reduced weight loss and delayed ripening (**Table 6.3**). Postharvest SA treatments in plums also showed significantly reduction of weight loss during storage (Davarynejad et al., 2015). Babalar et al. (2007) postulated that salicylic acid (SA) and its derivative ASA when applied pre- and postharvest to strawberry fruits decreased fruit metabolic activities, therefore decreasing fruit weight loss and carbohydrate depletion rate. A recent report further supported the beneficial effect of ASA foliar sprays in fruit trees, in this case leading to higher crop yield, quality parameters and bioactive compound content in pomegranate fruit (García-Pastor et al., 2020).

In the present study, ASA did not affect fruit growth. The effects of salicylic acid, or its derivatives such as ASA, on fruit growth has received less attention, compared with the studies on deriving resistance to biotic and abiotic stresses, due to its controversial role on plant growth depending on its concentration, climatic conditions and developmental stage applied (Rivas-San Vicente and Plasencia, 2011). In the present study, the ASA application was a one-off treatment at fruit-breaker stage, using a concentration from the upper limit used in other studies (Babalar et al., 2007; Sayyari et al., 2009; Lu et al., 2011; Giménez et al., 2014). Additional sprayings over the growing season may additionally be considered for future studies, particularly in growing seasons that induce more intense incidence of PS symptoms.

The commercial calcium formulation lowered PS incidence compared with the control (1.3% vs 2.4%) but not in a statistically significant manner. In Spain, calcium treatments are extensively used for controlling PS on loquat fruits during the change of

colour (Badenes, personal communication). Meda Ca is a commercial compound used to control calcium deficiencies and the product is recommended by the manufacturer to be used for other calcium-related disorders, as in the case of bitter pit in apples. Fruit treated with Meda Ca exhibited lower weight loss compared with control especially in H1 and H2 after maintenance at room temperature. However, whether this compound is more efficient compared to more economical calcium supplements that are routinely implemented (i.e. calcium nitrate) need to be dissected.

Spermidine did not affect fruit growth, while there are only few previous reports on Spd effect on fruit growth, yield parameters or preharvest performance (Jhalegar et al., 2012; Sharma et al., 2017). Spermidine provided a slight reduction in PS incidence, weight loss and some color parameters compared with control. However, considering its high application cost, it is not recommended for further experimentation in loquat orchards. Furthermore, no synergistic effect was monitored when spermidine was applied together with ASA. During fruit ripening and senescence, there is crosstalk between ethylene and PAs (as SAM is a common precursor for both growth regulators (Valero et al., 2002). The fact that loquat is a non-climacteric fruit may partially explain the reduced efficacy of spermidine treatment.

6.5.2 Effects of harvest time and storage

The registered % yield exhibiting the PS physiological disorder was relatively low compared with our previous studies on the same orchard that were implemented in other growing seasons (Hadjipieri et al., 2020b). Such data indicate the significance of meteorological conditions.

Meteorological data in the year of intense symptoms showed lower Tmin, Tmax and humidity values during January (prior to colour breaker stage) compared to the year of current experimentation (January 2019, data not shown). However, the year of intense PS symptoms was characterized by higher Tmin and Tmax values over the last on-tree developmental stages (after colour break) compared to the current year with low PS incidence. Based on that we develop the working hypothesis that cooler temperatures, before the fruit colour breaker stage, followed by higher temperatures can result in an accelerated harvesting season which leads to higher incidence of PS. Thus,

early harvesting periods favor PS symptoms in comparison to late harvest. This is also in accordance with previous findings, supporting that the severity of PS symptoms may substantially vary from year to year and it has been noted that early harvest favours the development of PS compared to late harvest (Gariglio et al., 2003).

Late-harvested fruit (H3) was of lower quality, depicted by differences in fruit size compared with H1 and H2. Moreover, fruit from the more advanced harvest exhibited lower FF, increased SSC, lower TA and increased a* values, in accordance with a previous study on the same cultivar (Hadjipieri et al., 2020a). CR symptoms were higher in the early harvest which may be related to the occurrence of unusually rainy period prior to the early harvest .

Yet a fruit that is considered a non-climacteric type and ripening procedure is concluded on the tree, soluble solids content increased during shelf life periods as elsewhere described (Hadjipieri et al., 2019; Hadjipieri et al., 2020a). Pinillos et al. (2011) reported that a minimum SSC value of 10° Brix, along with a preferable colour can be employed as harvest indicators, while Besada et al. (2013) mentioned an SSC values higher than 11° Brix ensures optimum consumer satisfaction. In our study, SSC values for all treatments were well above the threshold value of 11° Brix. During storage, RI increased, particularly at the more advanced harvests (Hadjipieri et al., 2020a). As the harvesting period was progressing, a scalar increase was noted for the RI. The RI registered elevated values after shelf life, particularly at advanced harvestings followed by 6 days of shelf life. Despite the fact that loquat fruit is considered as a non-climacteric type fruit (Tian et al., 2011), substantial changes on qualitative attributes occur that are not necessarily linked with fruit deterioration. Treatments, harvesting period and shelf life periods affected the loquat fruit quality parameters. As such, a disorder affecting the sweet/acid equilibrium can lead to presenting over-acid fruit to market, whereas absence of acidity can lead into marketing fruit of poor quality (Besada et al., 2013; Hadjipieri et al., 2020a).

The color parameter a*, indicating red coloration, as well as the a*/b* ratio increased during harvesting period and shelf life, the latter also positively affected the b* value. This is in accordance with our previous study (Hadjipieri et al., 2020a) where both a* and a*/b* parameters had higher values after shelf life periods. It was also noted that the higher values for the a*/b* ratio was manifesting deeper fruit coloration

compared with the freshly harvested fruits. Both Chroma and hue angle parameters as indicators of colour saturation showed an interesting trend with reference to the chemical treatments applied. Lower chroma values were noted for the ASA- and Spd-treated fruit. The hue angle measurements in our study were higher for all treatments in compare with control, whereas shelf life periods led to its reduction.

For this study the recommended 2 mM ASA preharvest foliar application has a cost of 104 €/ha for the chemical purchase, whilst the calcium treatment has a cost of 80 €/ha. The spermidine treatment cannot be recommended for use, as it is a costly treatment with no evident beneficial effect on loquat fruit quality parameters. The ASA lowered PS symptoms by 2.2% when compared with the control sample, which roughly equates to 1.86 kg per tree (average 600 trees per ha) of extra marketable fruit. Thus, with this treatment there is an added 1.1 tone of fruit that can be marketed and an average of 2500 € extra earnings per hectare, depended on harvesting season and fruit quality. This cost benefit analysis shows clear commercial upside with minimal outlay of capital expenditure and is therefore highly recommended for commercial use.

6.6 Conclusions

Loquat is an economically important tree crop with a highly perishable fruit; therefore, it is important to direct research towards methods to enhance fruit quality and postharvest performance. In the present study, foliar applications in cv. ‘Karantoki’ of an array of chemical compounds were applied. ASA was proven the most effective compound studied in both reducing PS appearance and enhancing some qualitative fruit properties. This treatment needs to be evaluated in growing seasons with intense symptoms of purple spot symptoms. Considering its low cost, the ASA application at multiple developmental stages, alone or in combination with a calcium supplement stands as an interesting future perspective.

7 The effect of modified atmosphere packaging on postharvest performance of two loquat cultivars

7.1 Abstract

Loquat (*Eriobotrya japonica* (Thunb.) Lindl) is the only fruit tree crop in the Mediterranean zone with ripening period during early spring, particularly when specific pre-harvest practices are applied such as cultivation under plastic cover. Loquat fruit is characterised by refreshing taste and it is highly appreciated by the consumers. However, loquat fruit is characterised by relatively short storage potential partially due to senescence and/or chilling related disorders. The aim of the current study was the evaluation of Xtend® packaging as a means of Modified Atmosphere Packaging (MAP) on postharvest performance of fruit from the main loquat cultivars grown in Cyprus, namely 'Karantoki' and 'Morphitiki'. Fruit were subjected to cold storage in conventional refrigerator (4°C) for 3 weeks and subsequently allowed at room temperature for 0, 2, 4 and 7 days, respectively. For each treatment, 30 fruits were used to determine weight loss, peel colour differentiations (CIEL*, a*, b*), flesh firmness (Texture analyser, Stable Micro Systems), soluble solids content (SSC) and titratable acidity (TA). 'Morphitiki' presented better phenotypic appearance compared to 'Karantoki' after extended storage, mainly due to reduced weight loss. Application of MAP significantly reduced weight loss after removal from cold storage. However, extended maintenance at room temperature (4 and 7 days) deteriorated the performance of both cultivars. As a technological perspective of the current study, data suggests that 'Morphitiki' fruit subjected to MAP can be refrigerated up to 3 weeks, provided they are consumed within two days after removal from storage.

Keywords: *Eriobotrya japonica*, cold storage, weight loss, soluble solids content, titratable acidity, chilling

7.2 Introduction

Loquat is a highly perishable commodity with relatively short shelf life period (Tian et al., 2007), while fruit can be cold stored at high relative humidity for up to 3 weeks (Kader, 2002). Due to its perishability, alternative methods to conventional cold storage (CS) with the aim to expand its market life were dissected such as: modified atmosphere packaging (MAP) (Ding et al., 2006; Pareek et al., 2014), controlled atmosphere (Ding et al., 2006), paper bags packaging and 1-methylcyclopropene (Zheng et al., 2005; Cao et al., 2011).

The minimum safe cold storage temperature ranges from 0-10 °C, highly dependent on the sensitivity of cultivar on chilling injury (CI) symptoms. Loquat fruits conditioned at 1 °C for 30 days in polyethylene bags with small holes kept their original quality and chemical characteristics intact (Ding et al., 1998). Cold storage at 5 °C in polyethylene bags of 20 μm width and with internal gas content 4 % O_2 and 5 % CO_2 showed better performance, while organic acids and carotenoid contents registered higher values compared to control (Ding et al. 2002).

Loquat cultivation in Cyprus is mainly based in two traditional cultivars, namely ‘Morphitiki’ and ‘Karantoki’ (**Figure 1.12**), for which relatively few data exist about their storage potential and postharvest performance. ‘Morphitiki’ cultivar has rounded to egg fruit shape, juicy white to yellow flesh with refreshing taste (Goulas et al., 2014). ‘Karantoki’ bears larger pear-shaped fruit and earlier fruit maturation (ca. 15 days earlier than ‘Morphitiki’). MAP treatment has been successfully applied in an array of commodities; in the current study its effect in loquat fruit was dissected.

7.3 Materials and Methods

7.3.1 Fruit material

Fruit material (cvs. ‘Morphitiki’ and ‘Karantoki’) were harvested from a covered commercial orchard (Episkopi, Limassol, Cyprus), based on size uniformity and external color. Fruit were initially used for the determination of physical dimensions §2.1. Subsequently fruit (control and MAP-treated) were placed into a conventional refrigerator at 4°C (CS) for 3 weeks and evaluated after 0, 2, 4 and 7 days shelf life,

respectively. The specific packaging is used worldwide for storing an array of commodities, such as pomegranates, cherries, bananas, fresh vegetables, citrus and stone fruits[23]. Each sub-lot was divided into three groups of 10 fruits

7.3.2 Methodology

All quality parameters are described in the relative chapters [Weight loss in § 2.2.1, color parameters (L*, a*, b*, a*/b*) in § 2.2.3, flesh firmness in §2.2.2, soluble solids content (SSC) in § 2.2.4 and titratable acidity (TA) in §2.2.6]. Ripening index (RI) was calculated as the SSC/TA ratio.

7.3.3 Statistical analysis

Data were analysed based on the ANOVA method according to 2×8-9 factorial design (2 cultivars × 8-9 treatments) with 30 replications for treatments combinations. Means were compared with the Duncan test at $P \leq 0.05$. All statistical analyses were carried out using the software package SPSS v22.0 (SPSS Inc., Chicago, USA).

7.4 Results and Discussion

Modified atmosphere packaging led to reduced weight loss (**Table 7.1**), in accordance with similar studies in other loquat cultivars (Amorós et al., 2008). However extended shelf life (4 or 7 days) after removal from cold storage led to substantially high weight loss in all cases. 'Karantoki' fruit showed higher weight losses after removal from cold storage, and intense shrivelling after extended shelf life period (**Figure 7.1**).

Flesh firmness registered higher values after removal from cold storage in both cultivars compared to harvest, both in control and MAP-treated fruit; 'Morphitiki' fruit presented higher firmness values than 'Karantoki' (**Table 7.1**). Notably, an increase in tissue firmness of specific loquat cultivars during postharvest has been reported, being attributed to lignin biosynthesis (Cai et al., 2006); while in other loquat cultivars, extended storage (6 weeks) led to firmness reduction (Amorós et al., 2008).

[23] DECCO-UPL 2005, www.deccoiberica.es/producto/bolsas-map/

Figure 7.1: Phenotypic appearance of 'Karantoki' (A) and 'Morphitiki' fruits (B) after 3-week refrigerated storage under Modified Atmosphere Packaging (MAP) and additional maintenance at room temperature (shelf life, SL) for 4 days.

Ripening index increased with the progress of shelf life period (**Table 7.1**). 'Karantoki' fruit possessed higher values, mainly due to lower titratable acidity, particularly after extended shelf life, as elsewhere described (Goulas et al., 2014). Both cultivars presented higher RI after CS with or without MAP treatment after shelf life of 7 days. Modifications in SSC were less intense compared to TA during the shelf life period for both cultivars (data not shown).

According to Amorós et al. (2008), L* values reduced in loquat fruit after CS treatments and subsequent maintenance at room temperature with only a slight decrease when the fruit was treated with MAP and then maintained at room temperature. The a* parameter showed an increase during SL after removal from CS, registering the highest values in 'Morphitiki' fruits after postharvest maintenance for 4 and 7 days, both in control and MAP-treated fruits. This is a typical phenomenon, also monitored in other loquat cultivars where the a* parameter was increased during SL after CS treatment (Amorós et al., 2008). The b* values went descending compared to harvest in both cultivars for both treatments. According to Amorós et al. (2008), the b* parameter showed a decrease during CS as well as CS and SL, while MAP treatment did not affect it. Notably, the ratio a*/b* in our study indicated that the 'Morphitiki' cultivar had a more desirable color which, over time, got deeper but was not affected by the MAP treatment as it showed no statistical difference in all corresponding treatments.

Table 7.1. Weight loss (%), ripening Index (SSC/TA), flesh firmness (g) of 'Karantoki' and 'Morphitiki' fruits at harvest and after refrigerated storage (cold storage, CS) with or without modified atmosphere packaging (MAP) and additional maintenance at room temperature (shelf life, SL) for 0, 2, 4 and 7 days, respectively. [24]

	Weight loss (%)		Ripening Index (SSC/TA)		Flesh firmness (g)	
Treatment	**Karantoki**	**Morphitiki**	**Karantoki**	**Morphitiki**	**Karantoki**	**Morphitiki**
Harvest			37.3±1.2g	19.3±2.5i	105.4±4.5f	130.1±7.2 cde
3 w CS + 0 d SL	3.5±0.2i	3.6±0.1i	67.6±5.0ef	35.7±8.8gh	125.3±4.7de	142.0±3.4 abcd
3 w CS + 2 d SL	7.5±0.4f	6.4±0.2g	77.9±1.5bcde	40.7±0.7g	142.0±9.6abcd	135.5±4.3 bcde
3 w CS + 4 d SL	8.7±0.3d	9.6±0.2e	83.6±2.0bcd	68.9±4.6ef	126.4±4.4 cde	133.5±4.9 bcde
3 w CS + 7 d SL	14.2±0.6a	10.5±0.3c	99.9±2.4a	76.6±2.1cde	119.0±6.8 ef	130.4±4.7 cde
3 w MAP + 0 d SL	0.7±0.04j	0.7±0.04j	63.1±3.07f	25.3±2.4fhi	145.3±5.6 abc	150.7±4.9 ab
3 w MAP + 2 d SL	5.0±0.2h	3.4±0.1i	77.8±4.6bcde	35.7±3.8gh	135.1±6.6 bcde	154.8±5.1a
3 w MAP + 4 d SL	9.3±0.3e	7.2±0.4f	88.3±2.1b	42.2±4.5g	139.2±5.5 abcd	150.6±5.4ab
3 w MAP + 7 d SL	15.0±0.5b	1.8±0.5d	86.2±3.82bc	73.9±0.6def	128.7±5.2 cde	141.7±3.1 abcd

[24] Results are the means ± standard error. The statistical analysis for each parameter shows the statistical difference between the two cultivars and the storage treatments.

Table 7.2: Color parameters for 'Karantoki' and 'Morphitiki' fruits at harvest and after refrigerated storage (cold storage, CS), with or without modified atmosphere packaging (MAP) and additional maintenance at room temperature (shelf life, SL) for 0, 2, 4 and 7 days, respectively. [25]

Treatment	L*		a*		b*		a*/ b*	
	Karantoki	**Morphitiki**	**Karantoki**	**Morphitiki**	**Karantoki**	**Morphitiki**	**Karantoki**	**Morphitiki**
Harvest	64.6±0.4b	67.7±0.3a	2.6±0.4jk	5.1±0.4efg	46.0±0.6b	48.5±0.6a	0.06ij	0.11gh
3 w CS + 0 d SL	49.8±1.1gh	48.4±1.2h	3.2±0.2ij	5.5±0.3def	38.7±0.8hi	36.9±0.7i	0.09hi	0.15cd
3 w CS + 2 d SL	53.8±0.9def	53.9±0.9def	4.1±0.2ghi	6.8±0.3b	40.5±0.7fgh	41.6±0.7efg	0.10gh	0.17bc
3 w CS + 4 d SL	54.7±0.8cdef	55.9±0.6cde	4.8±0.8efgh	8.1±0.3a	42.4±0.6def	43.8±0.6cde	0.11fg	0.19b
3 w CS + 7 d SL	54.9±1cde	53.1±1.2ef	6.2±0.3bcd	8.9±0.3a	44.5±0.8bcd	42.0±1.3ef	0.14def	0.22a
3 w MAP + 0 d SL	56.3±0.8cd	53.7±1.0def	2.8±0.2jk	5.6±0.4cde	42.1±0.6ef	40.3±0.7fgh	0.07ij	0.14def
3 w MAP + 2 d SL	51.8±0.9fg	49.0±1.3h	3.9±0.2hi	6.6±0.4bc	40.3±0.6fgh	38.9±0.8hi	0.10gh	0.17bc
3 w MAP + 4 d SL	49.2±1.0gh	7.4±0.8c	4.6±0.2efgh	8.1±0.4a	9 3±0.7gh	45.3 0.8b	0.12efg	0.18b
3 w MAP + 7 d SL	56.3±0.9cd	58.2±1.3cde	6.4±0.4bcd	8.9±0.4a	44.6±1.0bcd	42.6±1.4def	0.14cde	0.22a

[25] **. R**esults are the means ± standard error. The statistical analysis for each parameter (L*a*b* and a*/ b* ratio) shows the statistical difference between the two cultivars and the treatments.

7.5 Conclusions

Results indicated the beneficial effect of MAP in maintaining postharvest appearance of loquat fruit, even under extended conventional refrigerated storage, provided that they will be consumed in a short period after removal from storage. Furthermore, ‘Morphitiki’ fruits presented appreciable better appearance compared to ‘Karantoki’ fruit; the latter were characterized by high levels of weight loss that led to both quantitative and qualitative losses, particularly after extended shelf life periods. Therefore, the significance of genotype is also highlighted in the current study.

8 General conclusions

For the better understanding of the genotype significance, fruit qualitative attributes, phytochemical properties and incidence of physiological disorders during on-tree development and postharvest handling of loquat fruit (*Eriobotrya japonica* Lindl.) five independent, yet interrelated, experiments were contacted dealing with: (1) the carotenoid profile of loquat fruit during on-tree development, (2) the purple spot physiological disorder, (3) the effect of harvesting day on the phytochemical attributes, mechanical properties and postharvest performance of loquat fruit, (4) preharvest foliar spray applications in order to identify their efficacy on postharvest performance and (5) the effect of modified atmosphere on the postharvest performance of loquat fruit. Throughout the dissertation the following main conclusions derived, segregated into fundamental aspects and technological perspectives:

Fundamental aspects

- Carotenoid composition is greatly affected during on-tree loquat development with striking differences between peel and flesh tissue; 32 carotenoids were found in loquat peel, while only eighteen carotenoids were identified in the flesh.
- Trans-lutein and trans-β-carotene were the major carotenoids in the peel; the content of the former decreased with the progress of ripening, while the latter registered a substantial increase.
- Carotenoid profiling of loquat flesh indicated trans-β-cryptoxanthin, followed by trans-β-carotene and 5,8-epoxy-β-carotene to be the most predominant carotenoids.
- Elevated content of trans-β-carotene both in the flesh and in the peel with the progress of on-tree fruit development can be linked with the up-regulation of CYCB, a main carotenoid biosynthetic gene.

- Purple spot incidence is genotype-dependent and associated to sugar catabolism; ‘Obusa’, ‘Karantoki’ and ‘Morphitiki’ fruits exhibited severe, intermediate or no purple spot (PS) symptoms, respectively.
- Higher catabolism of sucrose to glucose is observed in loquat fruit peel with PS.
- *CWI3* and *VI* can be used as putative molecular markers for PS incidence, while *SuSy* registered the lowest expression levels in symptomatic tissue.

Technological prospectives

- Higher phytochemical content and antioxidant capacity is linked with early harvest.
- Bound phenolics are directly linked with the cell wall sugar moieties and account for 1/3 of total phenolic content.
- Acetylsalicylic acid is recommended as a cost-effective treatment that need to be further explored towards production of loquat fruit with enhanced properties.
- Acetylsalicylic acid and calcium supplement appear to control the incidence of purple spot physiological disorder.
- Spermidine did not show any significant beneficial effect on loquat fruit quality parameters.
- The beneficial effect of a commercially available MAP in maintaining postharvest appearance of loquat fruit after 3-week refrigerated storage was reported.
- ‘Morphitiki’ fruits presented appreciable better appearance compared to ‘Karantoki’ fruit after cold storage; the latter were characterized by high levels of weight loss that led to both quantitative and qualitative losses, particularly after extended shelf life periods.

These promising results can act as the foundation for further experimentation since all the aforementioned knowledge is expected to be furthered exploited in order to improve the overall quality of loquat production.

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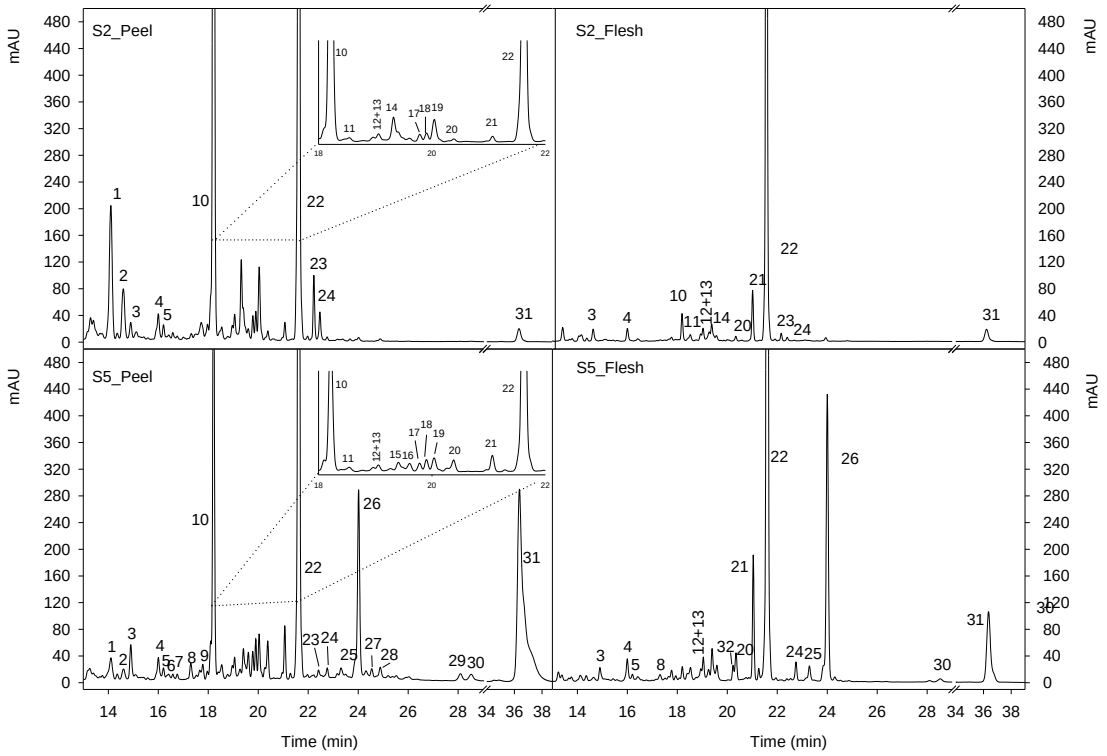
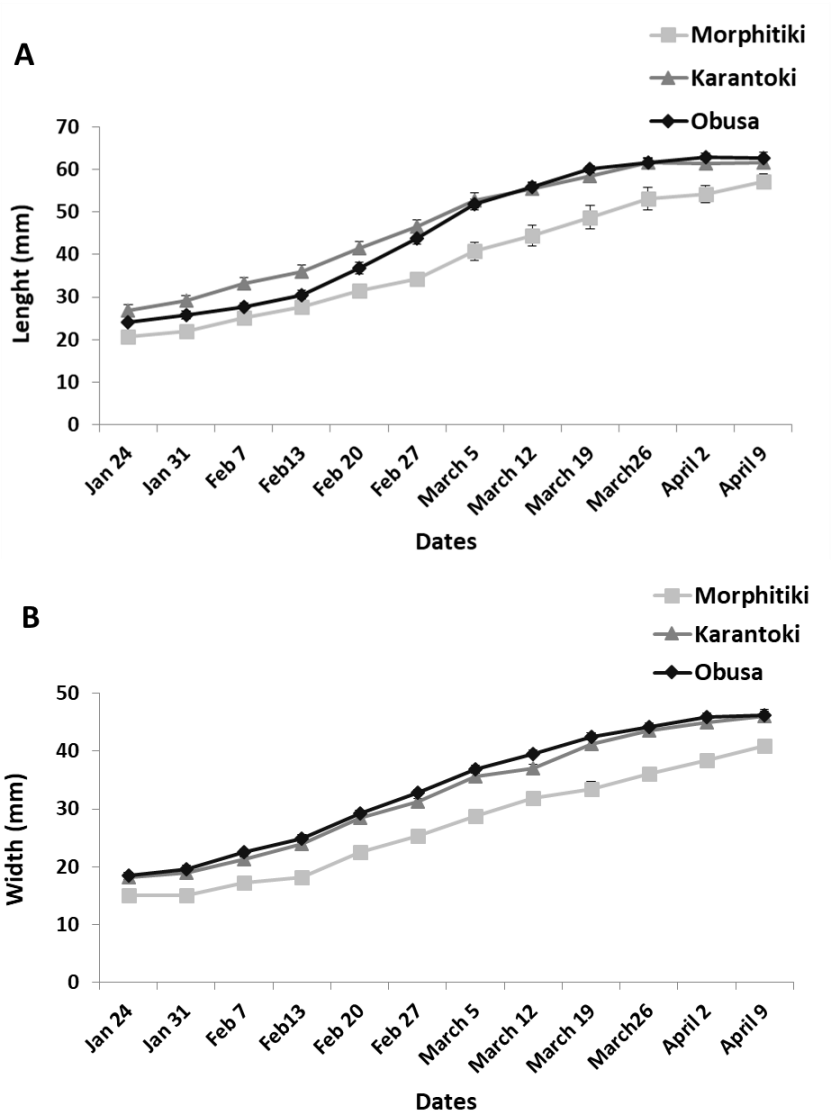
10 Supplementary Material



Supplementary Figure S1. HPLC chromatograms of saponified carotenoids in the peel and flesh of cv. 'Obusa' loquat fruits, at two maturity stages (S2 and S5) monitored at 450nm.



Supplementary Figure S2. Length (A) and width (B) of cvs. 'Morphitiki', 'Karantoki' and 'Obusa' during successive on-tree developmental stages. Data are the means of ten marked fruits ± SE.

**Figure elucidation was performed with GraphPad Software*

Supplementary Table S1. Previously published polyphenolic primers (*PAL1, PAL2, PPO1*) and carbohydrate primers (*CWI2, CWI3, SPS1, SPS2, NI2, NI3, SuSy, HXK, FRK, VI*), as well as housekeeping reference gene (*ACT*) used for real-time RT-qPCR analyses.

Gene	Reference	Primers	Nucleotide sequence 5'-3'	Ta (°C)
Actin (ACT)	Fu et al., 2012	*ACT-For*	AATGGAACTGGAATGGTCAAGGC	65
		ACT-Rev	TGCCAGATCTTCTCCATGTCATCCCA	
Phenylalanine ammonia lyase 1 (PAL1)	Shan et al., 2008	*PAL1-For*	TCGCAAGGCTACCTTGAAAC	60
		PAL1-Rev	GCATAAGATCCACTACAATGC	
Phenylalanine ammonia lyase 2 (PAL2)		*PAL2-For*	CCTGCATCATCGTCACTCAT	50
		PAL2-Rev	AACGGAATGGAATGCAGAAC	
Polyphenol oxidase 1 (PPO1)	Martínez-Márquez et al., 2013	*PPO1-For*	TACGACCAAGCCGGGTTCCC	65
		PPO1-Rev	TTCCACATTCGATCAACATTCGA	
Cell wall invertase 2 (CWI2)	Li et al., 2012	*CWI2-For*	TTCAAAGCTAAAGGCAGACACG	60
		CWI2-Rev	GTAAATCTACATCTACAAAGCCAGC	
Cell wall invertase 3 (CWI3)		*CWI3-For*	TAGACCATCATTTGCTGGCTATGT	50
		CWI3-Rev	TAAGGTGGGATAAACCCTGGATA	
Sucrose phosphate synthase 1 (SPS1)	Song et al., 2016	*SPS1-For*	GTTGTCCGCATCCCCTGTGG	65
		SPS1-Rev	GATCTCTTGCCTGGTGCTGGT	
Sucrose phosphate synthase 2 (SPS2)		*SPS2-For*	GATACTGGTGGTCAGGTTAAG	60
		SPS2-Rev	GTCTGCGTAATGCCATGGATG	
Neutral invertase 2 (NI2)	Li et al., 2012	*NI2-For*	GAGTTCCAGACAGGCATAAGGCT	60
		NI2-Rev	CCATCCGTCTATCAATCATACAGG	
Neutral invertase 3 (NI3)		*NI3-For*	GGTACTTGGTAGCGAAGATGATGT	60
		NI3-Rev	ACCAAACCCTTGTGCCGATTA	
Sucrose synthase (SuSy)	Zhou et al., 2006	*SuSy-For*	ACCCGATCCTTGCACTCTTGAGAA	65
		SuSy-Rev	GATCAGGCTTGCCATGCATTTCCT	
Hexokinase (HXK)	Qin et al., 2012	*HXK-For*	TAGCTACGTCGACAATCTCC	60
		HXK-Rev	CCGCAATGTAGTCAAAGAGT	
Fructokinase (FRK)	Qin et al., 2014	*FRK-For*	TAATGTTGCAGTTGGCATAG	60
		FRK-Rev	CTTGAAGCAACATATCAGCA	
Vacuolar invertase (VI)	Wang et al., 2015	*VI-For*	GGAAATTAGAAGGCGTATCGG	65
		VI-Rev	GCTGAGTCCGGATTGTACTG	

Supplementary Table S2. Weight (g), length (mm) and width (mm) of cv. 'Obusa' fruits during the on-tree developmental stages.

Maturity stage	Weight (g)	Length (mm)	Width (mm)
Immature green, S1	25.28±0.59 e	48.83±0.69 d	34.43±0.36 e
Mature green, S2	26.95±0.62 e	51.11±0.53 c	35.96±0.4 d
Breaker, S3	43.17±0.93 d	57.03±0.45 a	40.91±0.37 c
Half ripe, S4	50.95±1.23 c	55.91±0.66 ab	43.17±0.36 b
Fully ripe, S5	59.14±1.24 a	56.85±0.51 a	45.04±0.37 a
Over ripe, S6	55.28±1.05 b	54.82±0.48 b	44.05±0.36 ab

**Results are the mean ± standard error (n=10). The statistical analysis for each parameter (weight, length and width) shows the statistical difference between the six developmental stages.*

Supplementary Table S3. Colour measurements for peel and flesh of cv. 'Obusa' fruits, over six developmental stages, expressed as L*a*b* values and a^*/b^* ratio.

Maturity stage	Peel			
	L*	**a***	**b***	**a*/ b***
Immature Green, S1	45.65±0.75e	-16.73±0.22e	28.20±0.75e	-0.60f
Mature Green, S2	52.56±0.69d	-17.66±0.26f	36.06±0.77d	-0.50e
Breaker, S3	64.99±0.37a	-9.81±0.38d	49.19±0.34a	-0.20d
Half ripe, S4	58.35±0.70c	3.22±0.29c	43.47±0.57b	0.08c
Fully ripe, S5	58.78±0.62c	10.01±0.3b	41.52±0.53c	0.24b
Over ripe, S6	60.61±0.61b	12.42±0.27a	43.58±0.58b	0.29a
	Flesh			
Immature Green, S1	74.32±0.56a	-11.51±0.33f	36.05±0.53a	-0.32f
Mature Green, S2	66.70±0.58b	-5.69±0.31e	30.87±0.63b	-0.19e
Breaker, S3	61.33±1.07c	-1.84±0.19d	30.75±0.6b	-0.06d
Half ripe, S4	52.12±1.32d	3.95±0.26c	32.37±0.64b	0.12c
Fully ripe, S5	53.62±0.77d	9.33±0.24b	34.94±0.48a	0.27b
Over ripe, S6	53.46±0.69d	10.67±0.23a	36.67±0.52a	0.29a

** The statistical analysis for each parameter (L*a*b* and a^*/b^* ratio) shows the statistical difference between the six developmental stages.*

Supplementary Table S4. UV/vis spectra and characteristic ions of carotenoids from six maturation stages of loquat fruits, obtained by HPLC-PDA-MS.

Peak	Carotenoid	t_R (min)	λ_{max} (nm)	%III/II	%Ab/II	[M+H]+ m/z	HPLC/APCI(+)-MS^n experiment *m/z* (% base peak)	Exact mass	Score	Error (ppm)	Molecular formula
1	All-*trans*-neoxanthin	14.1	412,436,462	70	0	601 (40), 583 (75), 565 (100)	MS^2 [601]: 583 (100), 565 (47), 547 (9), 509 (6),491 (5), 221 (41) MS^3 [601>583]: 565 (48), 547 (14)	600.4188	91.88	-2.69	$C_{40}H_{56}O_4$
2	All-*trans*-neochrome	14.6	397,420,448	90	0	601 (42), 583 (100), 565 (43)	MS^2 [601]: 583 (100), 565 (57), MS^3 [601>583]:221 (62)	600.4178	96.70	-0.89	$C_{40}H_{56}O_4$
3	All-*trans*-violaxanthin	14.8	414,438,470	75	0	601(85), 583(100), 565(20)	MS^2 [601]: 583 (100), 565 (12), 509 (5), 221 (24)	600.4178			$C_{40}H_{56}O_4$
4	Not identified	16.0	466	0	0	455 (100)	MS^2 [455]: 437 (80), 399 (34)	455.3324	93.60	-2.61	$C_{33}H_{43}O$
5	Not identified	16.2	396,420,448	75	0	601(60)	MS^2 [601]: 583(95), 221(100				
6	Not identified	16.4	No detected	--	--	601(64)	MS^2 [601]: 583 (90), 565 (40), 491 (9), 221 (100) MS^3 [601>583]:565 (100),221 (60)				
7	β- Diepoxy-cryptoxanthin	16.7	412, 436,466	72	0	585(100)	MS^2 [585]: 567 (45), 549 (80), 493 (37), 221 (100), 205 (10)	584.4184	95.60	-2.52	$C_{40}H_{56}O_3$
8	*Cis*-violaxanthin	17.3	324,410, 434,464	60	8	601(81), 487(100)	MS^2 [601]: 583 (100), 565 (42), 509 (6),491 (23), 221 (51) MS^2 [487]: 469(100)				
9	Not identified	17.8	378,400,424	100	0	601(100), 351(98)	MS^2 [601]: 583 (100), 565 (13), 509 (15), 491 (14),393 (40),221 (41)				

							MS^2 [351]: 333(16)				
10	All-*trans*-lutein	18.2	420,444,472	48	0	569 (5), 551 (100)	MS^2[551]: 533(51), 495(24), 477(35)	568.427	99.30	-0.226	$C_{40}H_{56}O_2$
11	Not identified	18.5	444	0	0	454(100)	MS^2[454]: 436(7), 393(100)				
12	Not identified	18.9	448	0	0	473(100),539(80)	MS^2[539]: 521(100)				
13		19.0	454	0	0	473 (100), 454 (14)	MS^2 [473]: 455(43), 205(100) MS^2 [454]: 436(31), 393(36)				
14	Not identified	19.3	423,438,474	60		551(100)	MS^2 [551]: 533(67), 477(55)				
15	Not identified	19.4	448	0	0	439(93), 403(100)	MS^2 [439]:403(14)				
16	Not identified	19.6	434	0	0	391(100)	MS^2 [391]:373(14)				
17	5,6-Epoxy-β-cryptoxanthin	19.8	416,438,466	34	0	569 (17),551 (54)	MS^2 [551]: 533 (20), 205(21)	568.428	75.56	-5.07	$C_{40}H_{56}O_2$
18	5',6'-Epoxy-β-cryptoxanthin	19.9	419,441,470	52	0	569(30), 551(100)	MS^2 [551]: 533(100), 577(14), 459(30), 221(13)	568.428	89.42	-2.11	$C_{40}H_{56}O_2$
19	*Cis*-lutein	20	326,412,436,462	52	20	569(18), 551 (100)	MS^2 [551]: 533(100), 221(11)	568.428	77.7	3.57	$C_{40}H_{56}O_2$
32	β- Diepoxy-cryptoxanthin	20.2	416,440, 470	87	0	585(100)	MS^2 [583]: 567(49), 549(10) 221(31), 205(29)				
20	Not identified	20.4				446(100) 417(90)	MS^2 [417]:399(100) MS^2 [446]:219(100)				
21	β-Apo-8'-carotenal (IS)	21.0	450	0	0	417(100)	MS^2 [417]:399(25), 389(32), 361(39), 325(100), 293(97), 157(88), 119(16)	416.3079	98.23	-2.15	$C_{30}H_{40}O$
22	Citranaxanthin	21.6	470	0	0	457(100)	MS^2 [457]:439(90), 399(49)	455.3324	97.23	-1.25	$C_{33}H_{44}O$
23	Not identified	22.4	470			696(100)	MS^2 [696]: 534(34), 516(100)				
24	Not identified	22.8	420,444,470	31	0	537 (58)	MS^2 [537]: 467 (16), 444 (51), 365 (100)				

25	Not identified	23.3	Espectro raro			537(28),430(100)	MS^2 [537]:444(100),481(24) 413(90)				
26	All-*trans*-β-cryptoxanthin	24.0	420, 448,472	24	0	553(100)	MS^2 [553]:535(100), 497(41),461(10)				
27	Not identified	24.5				664(100)	MS^2 [664]: 551(100), 496(55)				
28	Not identified	24.9	453, 479	0	0	551(100)	MS^2 [551]: 534(15), 361(100)				
29	Phytoene+ not identified 14	28.0				545(95), 553(100)	MS^2 [545]:489 (10),395 (100) MS^2 [553]:535(12)				
30	5,8-epoxy-β-carotene	28.4	405, 424, 450	26	0	553(100)	MS^2 [553]:535 (55),461 (35), 221(64),205(17)				
31	All-*trans*-β-carotene	36.5	424, 446,470	18	0	537	MS^2 [537]:444 (100)				

Supplementary Table S5. Quantification of the identified carotenoids (Supplementary Table S4) in peel and flesh for six maturity stages in loquat. Results are expressed as µg/100 g fresh weight. Standard deviations (n=3) in parentheses.

Peak nº	**Maturity stages (peel)**						**Maturity stage (flesh)**					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
1	44.3 (1.5)	27.9 (3.3)	24.4 (4.7)	25.1 (2.4)	26.9 (1.2)	25.9 (2.9)	13.8 (2.1)	10.9 (0.4)	10.0 (0.8)	13.3 (0.2)	--	--
2	26.1 (2.6)	23.3 (1.3)	16.4 (1.7)	23.9 (2.1)	22.9 (1.3)	22.4 (0.2)	13.6 (2.1)	11.5 (0.1)	10.5 (0.6)	13.1 (0.2)	--	--
3	15.6 (2.5)	17.6 (0.3)	16.4 (1.7)	18.9 (1.2)	26.8 (3.3)	25.2 (4.6)	--	--	--	12.5 (0.2)	18.1 (0.8)	21.5 (1.6)
5	17.3 (2.0)	16.6 (0.9)	15.4 (0.0)	17.9 (0.6)	21.4 (0.8)	21.9 (1.4)	--	--	--	--	16.3(0.4)	19.2 (1.5)
6	--	16.0 (0.8)	14.6 (0.8)	17.5 (1.0)	21.0 (1.1)	21.3 (0.4)	--	--	--	--	--	--
7	--	--	15.0 (0.8)	17.3 (0.8)	21.5 (1.8)	21.2 (1.7)	--	--	--	--	--	--
8	16.2 (2.45)	15.9 (0.6)	14.8 (0.2)	17.7 (0.7)	23.3 (2.0)	23.2 (3.0)	--	--	--	12.3 (0.1)	16.5 (0.9)	19.1 (1.5)
9	19.2 (1.9)	--	17.4 (0.8)	19.9 (1.2)	23.1 (1.6)	22.8 (0.9)						
10	1621.5 (12.2)	1228.6 (285.4)	1241.5 (150.1)	1068.2 (162.1)	774.3 (70.4)	688.4 (70.3)	24.1 (6.0)	11.5 (3.7)	--	--	--	--
17	16.9 (2.7)	19.5 (2. 9)	20.2 (3.7)	21.4 (1.3)	24.6 (1.9)	23.1 (0.2)	---	--	--	--	--	--
18	19.4 (3.0)	19.6 (2.4)	21.0 (3.9)	23.9 (2.0)	23.7 (0.9)	25.4 (0.7)	---	--	--	--	--	--
19	26.3 (1.8)	23.7 (0.6)	20.5 (1.9)	23.1 (2.9)	26.1 (1.4)	29.6 (2.8)	---	--	--	--	--	--
24	85.3 (1.2)	82.4 (5.7)	72.3 (4.3)	80.3 (3.9)	98.8 (5.7)	98.4 (2.3)	--	--	--	56.3 (0.3)	82.1 (4.1)	100.1 (6.2)
25	68.6 (2.3)	73.5 (7.0)	67.8 (4.3)	80.6 (4.2)	101.9 (5.8)	105.5 (1.9)	--	--	--	62.1 (0.3)	84.4(5.6)	95.7 (6.80)
26	16.3 (2.2)	16.1 (1.1)	19.0 (6.2)	27.2 (6.7)	54.3 (5.0)	58.4 (6.6)	--	--	--	65.4 (6.4)	329.0 (13.3)	595.0 (25.0)

30	--	--	67.4 (4.4)	81.6 (2.7)	106.5 (6.5)	109.9 (5.8)	--	--	--	56.0 (1.3)	75.9 (3.0)	87.8 (6.3)
31	151.9 (30.9)	188.7 (46.4)	473.7 (42.2)	502.8 (23.8)	897.8 (54.5)	1096.9 (38.7)	80.9 (12.7)	77.7 (4.0)	124.6 (10.2)	187.7 (30.9)	253.2 (19.0)	218.2 (48.2)
32	--	--	--	--	--	--	--	--	--	--	17.6(1.2)	21.4(1.8)

Supplementary Table S6. *Relative expression levels of polyphenolic biosynthetic genes* (PAL1, PAL2, PPO1) *and carbohydrate biosynthetic genes* (CWI2, CWI3, SPS1, SPS2, NI2, NI3, SuSy, HXK, FRK, VI) *in loquat fruit peel of cvs. 'Karantoki' and 'Obusa' on both symptomatic (30-50%) and asymptomatic peel tissue at fully-ripe stage (n = 3). Values that differ from the control (cv. 'Morphitiki' asymptomatic peel tissue at fully-ripe stage) with significance level P ≤ 0.05 are marked with bold letters. Data are based on a statistical analysis of the means of three biological replications (Pfaffl et al., 2002).*

	Genes												
Developmental stages	***PAL1***	***PAL2***	***PPO1***	***CWI2***	***CWI3***	***SPS1***	***SPS2***	***NI2***	***NI3***	***SuSy***	***HXK***	***FRK***	***VI***
Karantoki asymptomatic	**-1.629**	**2.288**	**-1.978**	-1.629	-1.159	1.211	1.125	1.006	**1.753**	-1.464	1.295	1.074	-1.396
Obusa asymptomatic	**4.036**	**1.347**	-1.385	**-4.891**	1.127	1.141	1.508	1.219	**2.388**	-1.667	1.319	**2.614**	1.013
Karantoki with Purple Spot	-1.314	**2.378**	1.286	**-2.491**	-1.169	**3.173**	1.117	1.433	**2.450**	**-2.164**	1.319	1.334	-1.184
Obusa with Purple Spot	**3.380**	1.189	1.644	**-21.496**	**1.717**	1.621	1.170	1.347	1.212	**-2.351**	1.107	**2.124**	**2.114**
	Genes and p-values												
Developmental stages	***PAL1***	***PAL2***	***PPO1***	***CWI2***	***CWI3***	***SPS1***	***SPS2***	***NI2***	***NI3***	***SuSy***	***HXK***	***FRK***	***VI***
Karantoki asymptomatic	**0.0220**	**0.0010**	**0.0010**	0.1855	0.5315	0.7445	0.6370	0.9380	**0.0455**	0.5490	0.4400	0.8280	0.5670
Obusa asymptomatic	**0.0445**	**0.0010**	0.4495	**0.0010**	0.4055	0.7825	0.0990	0.5715	**0.0455**	0.3950	0.3300	**0.0010**	0.9645
Karantoki with Purple Spot	0.6325	**0.0010**	0.5080	**0.0010**	0.3115	**0.0010**	0.7935	0.3595	**0.0010**	**0.0500**	0.4760	0.3915	0.6945
Obusa with Purple Spot	**0.0445**	0.3900	0.4660	**0.0010**	**0.0250**	0.2485	0.5715	0.3985	0.5495	**0.0010**	0.7405	**0.0010**	**0.0250**

Supplementary Table S7. Effects of pre-harvest foliar applications with different chemicals on percentage (%) of total yield in different fruit quality categories (Extra, A, B, PS, CR) collected during three harvesting dates. LSD: 1.798

	Total yield (%)														
	Harvest 1 (5th April)					Harvest 2 (19th April)					Harvest 3 (6th May)				
	Extra	**A**	**B**	**P S**	**CR**	**Extra**	**A**	**B**	**P S**	**CR**	**Extra**	**A**	**B**	**P S**	**CR**
Control	71.7±3.7	20.9±4.3	0.4±0.1	2.0±1.0	5.2±0.7	72.5±8.9	21.4±7.0	3.9±2.4	1.7±1.0	0.5±0.2	54.4±2.2	35.1±4.1	6.2±1.7	4.1±3.3	0.3±0.2
15% w/w Meda Ca	76.0±8.4	19.4±8.5	1.4±0.7	0.6±0.6	2.6±0.9	83.0±2.3	13.5±3.0	1.0±0.3	1.6±0.8	1.2±0.5	71.5±2.9	23.1±2.5	3.2±0.3	1.4±0.7	0.8±0.1
2 mM Spd[1]	76.3±4.6	16.3±2.8	0.5±0.3	1.0±0.3	5.8±1.6	75.3±6.5	20.1±5.8	2.0±1.4	2.0±1.2	0.7±0.1	67.2±3.9	27.5±3.5	4.3±0.6	0.8±0.4	0.3±0.2
2 mM ASA[2]	66.1±7.5	26.0±6.2	2.4±1.2	0.9±0.2	5.3±0.7	78.1±4.3	17.9±2.8	2.7±1.2	0.2±0.0	1.1±0.2	47.4±3.3	40.9±2.6	10.5±2.7	0.4±0.4	0.8±0.8
2 mM ASA + 2 mMSpd	69.5±4.2	23.2±2.6	2.0±0.8	0.8±0.8	4.5±1.5	68.8±2.9	26.2±2.3	3.8±0.6	0.6±0.2	0.6±0.2	56.8±1.9	32.7±2.2	8.9±1.2	1.3±0.9	0.2±0.2

[1], Spermidine
[2], Acetylsalicylic Acid

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Metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway in peel and flesh tissue of loquat fruit during on-tree development

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Abstract

Background: Carotenoids are the main colouring substances found in orange-fleshed loquat fruits. The aim of this study was to unravel the carotenoid biosynthetic pathway of loquat fruit (cv. 'Obusa') in peel and flesh tissue during distinct on-tree developmental stages through a targeted analytical and molecular approach.

Results: Substantial changes regarding colour parameters, both between peel and flesh and among the different developmental stages, were monitored, concomitant with a significant increment in carotenoid content. Key genes and individual compounds that are implicated in the carotenoid biosynthetic pathway were further dissected with the employment of molecular (RT-qPCR) and advanced analytical techniques (LC-MS). Results revealed significant differences in carotenoid composition between peel and flesh. Thirty-two carotenoids were found in the peel, while only eighteen carotenoids were identified in the flesh. *Trans*-lutein and *trans*-β-carotene were the major carotenoids in the peel; the content of the former decreased with the progress of ripening, while the latter registered a 7.2-fold increase. However, carotenoid profiling of loquat flesh indicated *trans*-β-cryptoxanthin, followed by *trans*-β-carotene and 5,8-epoxy-β-carotene to be the most predominant carotenoids. High amounts of *trans*-β-carotene in both tissues were supported by significant induction in a chromoplast-specific *lycopene β-cyclase (CYCB)* transcript levels. *PSY1*, *ZDS*, *CYCB* and *BCH* were up-regulated and *CRTISO*, *LCYE*, *ECH* and *VDE* were down-regulated in most of the developmental stages compared with the immature stage in both peel and flesh tissue. Overall, differential regulation of expression levels with the progress of on-tree fruit development was more evident in the middle and downstream genes of carotenoid biosynthetic pathway.

Conclusions: Carotenoid composition is greatly affected during on-tree loquat development with striking differences between peel and flesh tissue. A link between gene up- or down-regulation during the developmental stages of the loquat fruit, and how their expression affects carotenoid content per tissue (peel or flesh) was established.

Keywords: *Eriobotrya japonica*, Developmental stages, Maturation, Ripening, β-carotene, β-cryptoxanthin, Lutein, LC-MS, Biosynthetic pathway

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Background

Loquat (*Eriobotrya japonica* Lindl) is a member of the Rosaceae family that is commercially cultivated in many countries [1–3], being highly appreciated for its light, refreshing taste [2, 4]. Therefore, although initially considered as an underutilized crop, nowadays loquat can gain added value as it is available during late winter-early spring period [5]. The loquat tree has three flushes of growth per year, and the principal tree growth can be separated into 8 distinct developmental stages [2]. In particular, under Mediterranean weather conditions, the tree blooms between October and early November and its fruit develops through winter, ripening from early February until May. Fruit is usually consumed fresh, but it is also known to be used processed into jam, jelly, wine, syrup, and juice. It is also known that the leaves, flowers and fruits are traditionally used in Chinese medicine since they are linked with health-promoting properties [1, 6].

Carotenoids play an important role in loquat, as they affect organoleptic characteristics and health properties of the fruit. In particular, carotenoids are the main pigments in loquat and impact flavor acceptability, since they are precursors of important volatile flavor compounds [7]. Regarding nutraceutical properties of fruit carotenoids, a significant number of studies depicted their beneficial effect to the promotion of health, including the prevention and/or treatment of chronic and cardiovascular diseases [8]. In particular, fruits rich in carotenoids are directly connected to the prevention of inflammation and cataract [1, 9, 10] and are also known to enhance immune responses [1, 9, 11]. Carotenoid profile in loquat is influenced by maturity stage, environmental and most promptly genetic factors. Loquat cultivars have been segregated to white- and red-fleshed [10, 12]. However, segregation of loquat cultivars based on their flesh color can be confusing, since additionally the terms yellow- and orange-fleshed are being used. White-fleshed cultivars have a creamy, pale yellow color, while the terms red- and orange-fleshed can be considered as synonymous. The latter type cultivars have higher carotenoid concentrations than the lighter coloured ones [1, 5, 9, 10, 12].

Carotenoids are formed from isopentenyl diphosphate (IPP), a five-carbon compound, and dimethylallyl diphosphate (DMAPP), its allylic isomer. These compounds form geranylgeranyl diphosphate (GGPP) which in turn forms phytoene through the activity of *phytoene synthase (PSY)*. Phytoene forms lycopene via four desaturation reactions with the involvement of *phytoene desaturase (PDS) ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS)* and *carotene isomerase (CRTISO)* [13]. Lycopene, in turn undergoes a series of reactions to form lutein, through the ε, β-branch, the predominant carotenoid pigment in green plants [13], and violaxanthin from zeaxanthin with the presence of *zeaxanthin epoxidase (ZEP)* though the β, β-branch (Fig. 1). This forms the xanthophyll cycle, the mechanism that enables plant adaptation to high light stress [10]. 9-cis-neoxanthin is derived from the conversion of violaxanthin by *neoxanthin synthase (NSY)*, which in turn forms the phytohormone abscisic acid through *9-cis-epoxycarotenoid dioxygenase (NCED)* activity [11, 14], which controls abiotic stress signaling pathways [14].

The carotenoid biosynthetic pathway is controlled by the presence of the key enzyme PSY (Fig. 1, [10, 11, 13, 15–17]). Regarding loquat fruit, Fu et al. [10] investigated the mechanism underlying the differentiation of carotenoids in a red-fleshed (cv. 'Luoyangqing') and a white-fleshed (cv. 'Baisha') cultivar; differences in carotenoid accumulation in the two cultivars were linked with the differential expression of *PSYI*, *CYCB,* and *BCH* genes. The aim of the current study was to monitor the carotenoid composition in peel and flesh tissue of 'Obusa' fruits, an orange-fleshed cultivar, in correlation with the progress of fruit maturity. Towards this aim, high-resolution temporal expression profiles of carotenoid biosynthetic genes in both tissues were determined by RT-qPCR and linked with individual carotenoids, quantified by LC-MS.

Methods

Fruit material and experimental design

Loquat fruits cv. 'Obusa' were harvested at ca. 10-day intervals, between March 30th and May 14th (Fig. 2), from a commercial orchard (Episkopi, Lemesos, Cyprus), owned by the first author. For each developmental stage, 30 uniform fruits were selected based on size and colour; such fruit were divided into three 10-fruit sublots, representing the biological replications. Fruit were initially used for the determination of physical dimensions and colour and subsequently for molecular and analytical analysis, as described below. The developmental stages were defined using the BBCH scale [2].

For the molecular analysis, fresh samples of both peel and flesh were flash frozen in liquid nitrogen and maintained at −80 °C until needed. For the determination of carotenoid profiles, samples were freeze-dried (Freeze Dryer-Christ Alpha 1–4 LD plus).

Quality attributes

Physical dimensions of fruit were determined with the employment of an analytical grader and an electronic calibre (IS11112, Insize). The colour parameters CIE L* (brightness or lightness; 0 = black, 100 = white), a* (−a* = greenness, +a* = redness) and b* (−b* = blueness, +b* = yellowness) were measured at the peel and at the flesh tissue per fruit with a chroma meter (CR-400, Konica Minolta).

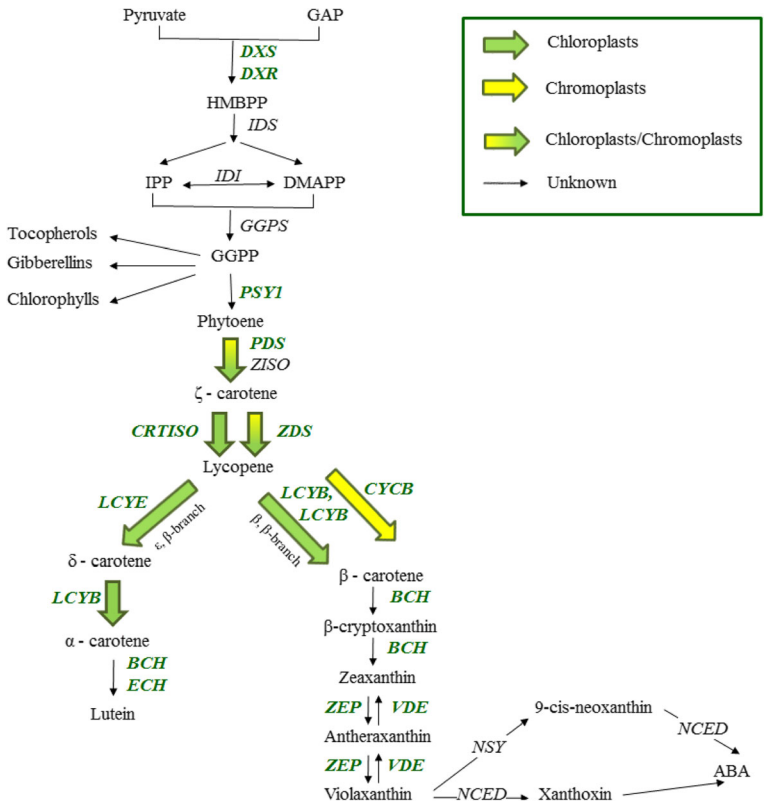

Fig. 1 Carotenoid biosynthetic pathway in loquat fruit. Genes examined are in *green italic bold letters*. The enzymes/genes are: DXS, 1-deoxy-D-xylulose 5-phosphate-synthase; DXR, DXP reductoisomerase; IDS, isopentenyl pyrophosphate synthase; IDI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl diphosphate synthase; PSY1, phytoene synthase; PDS, phytoene desaturase; ZISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene β-cyclase; CYCB, chromoplast-specific lycopene β-cyclase; LCYE, lycopene ε-cyclase; BCH, β-carotene hydroxylase; ECH, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED 9-cis-epoxycarotenoid dioxygenase. The metabolites are: pyruvate; GAP, D-glyceraldehyde 3-phosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GGPP, geranylgeranyl diphosphate; Phytoene; ζ – carotene; lycopene; α – carotene; β – carotene; δ - carotene; lutein; β-cryptoxanthin; zeaxanthin; antheraxanthin; violaxanthin; 9-cis-neoxanthin; xanthoxin; ABA, abscisic acid (Figure is modified from [10, 11, 13, 16, 17])

Spectrophotometric determination of carotenoid and chlorophyll content

Twenty mL of acetone–hexane (4:6, *v*/v) were added to 100 mg of lyophilised plant material and thoroughly mixed. Upon separation of the two phases, the absorbance was determined in the supernatant at 453, 505, 645 and 663 nm. Based on Nagata and Yamashita equations, total carotenoids, chlorophyll-a and chlorophyll-b contents were determined [18].

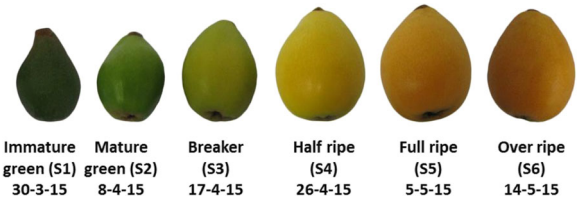

Fig. 2 Phenotypes of loquat fruits during six distinct on-tree developmental stages (S1-S6)

Liquid chromatography mass spectrometry (LC-MS) analysis of carotenoids

Extraction and saponification of loquat carotenoids

Liquid-liquid extraction and saponification of the samples was carried out as previously described by Minguez-Mosquera & Hornero-Mendez [19]. Briefly, 0.5 g of freeze-dried tissue (peel or flesh) was homogenized in acetone-butylated hydroxytoluene - 0.1% using an UltraTurrax (Ika, Staufen, Germany) and centrifuged (2000 rpm, 10 min, 4 °C). Extraction steps were repeated until complete removal of colour in the sample. The internal standard used was β-Apo-8′-carotenal (Sigma, St Louis, MO, USA). The extracts were combined and treated with diethyl ether. A solution of NaCl (10%, *w*/*v*) was added to separate the phases. The lower phase was discarded and the remaining phase was washed with Na_2SO_4 (2%, *w*/*v*) to remove water residues. Fifty mL of a methanolic solution of KOH (20%, *w*/*v*) was added and left for 1 h in darkness. The organic phase was washed

several times with deionized water until washings were neutral. It was then filtered through a bed of anhydrous Na_2SO_4 and evaporated until dry using a speed vacuum (Thermo Scientific Savant SPD121P). The pigments were collected with 1 mL of acetone: methanol (7:3, *v*/v) and stored at −20 °C until needed. To prevent isomerization and photodegradation of carotenoids, all procedures were carried out under pale light.

LC- MS analysis of loquat carotenoids

The carotenoid analysis was carried out using an Agilent 1200 HPLC equipped with a photodiode array detector and a single quadrupole mass spectrometer detector in series (6120 Quadrupole, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a reverse-phase Poroshell 120 EC-C_{18} column (100 mm × 3 mm, 2.7 μm particle size) (Agilent Technologies) operating at 32 °C. Water with 0.05 M ammonium acetate and acetonitrile: methanol (70:30) were used as mobile phases A and B, respectively, with a flow rate of 0.7 mL min^{-1}. The linear gradient started with 60% of solvent B in A, reaching 100% solvent B at 20 min; this was maintained up to 35 min. The initial conditions were re-established at 36 min and kept under isocratic conditions up to 40 min. Injection volume was 5 μL. Detection and quantification of all carotenoids and carotenoid esters were carried out in UV-vis at 450 nm (Additional file 1: Figure S1).

The identification of carotenoids in loquat was carried out on an Agilent 1100 HPLC system equipped with a photodiode array and an ion trap mass spectrometer detector (Agilent Technologies, Waldbronn, Germany). The mass detector was a Bruker ion trap spectrometer (model HCT Ultra) equipped with an APCI (Atmospheric Pressure Chemical Ionization). The mass spectrometer parameters were as follows: positive ion mode (APCI +); source temperature, 350 °C; probe temperature, 450 °C, corona voltage, 4.0 kV; The full scan mass covered the range from *m/z* 100 up to *m/z* 1200 and the target mass was adjusted to 350. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the positive mode, and the MS^n was carried out in the automatic mode. The identification of the peaks was performed by the extracted ion-chromatograms of the ion current at *m/z* values corresponding to the $[M-H]^+$ ions of the individually investigated compounds, as well as their fragmentation. Furthermore, to confirm the identification of the carotenoids and obtain a more reliable identification, samples were analyzed with the Agilent 1290 Infinity UPLC system coupled to a quadrupole (Q-TOF) mass spectrometer (6550 Accurate-Mass QTOF, Agilent Technologies) using an electrospray interface with jet stream technology. The chromatographic separation was developed under the same conditions, as described above. The optimal conditions for the electrospray interface were as follows: gas temperature: 300 °C, drying gas 11 L/min, nebulizer 65 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. Spectra were acquired in the *m/z* range of 100–1100, in a positive mode and with an acquisition rate of 1.5 spectra in MS, maintaining a mass resolution over 50,000 for the mass range used. Internal mass calibration by the simultaneous acquisition of reference ions and mass drift compensation was used for obtaining low mass errors. Q-TOF MS data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00). For quantification, β-apo-8′-carotenal was used as an internal standard. Lutein, β-carotene and violaxanthin (Sigma, St Louis, MO, USA) in a concentration range of 5–100 $\mu g.mL^{-1}$ were used to quantify compounds in three different groups, hydroxycarotenoids, carotenes, and epoxycarotenoids, respectively. Neoxanthin, neochrome, β-cryptoxanthin epoxides and β-carotene epoxides were estimated as violaxanthin. β-Cryptoxanthin was quantitated as lutein. The *cis*-isomers were quantitated with the calibration curve of the all-*trans* isomers. Concentrations were expressed as micrograms of pigment per 100 g of sample fresh weight (μg 100 g^{-1} F.W.).

RNA extraction, cDNA synthesis and quantitative real-time RT-PCR analysis

Total RNA was extracted from three bulked biological replicates of loquat fruit material for each developmental stage (S1-S6) according to a modified cetyltrimethylammonium bromide (CTAB) protocol developed by Gambino et al. [20]. Next, RNA integrity was confirmed spectrophotometrically (Nanodrop 1000 Spectrophotometer, Thermo Scientific) followed by gel electrophoresis and samples were treated with RNase-free DNase (Cat. No. NU01a, HT Biotechnology LTD, England) to remove total gDNA, as elsewhere described [21].

Total RNA (0.5 μg) was reverse transcribed using the PrimeScript™ RT reagent Kit (Takara Bio, Japan), according to the manufacturer's protocol (Takara Bio, Japan). Subsequently, quantitative real-time RT-PCR was performed with BioRad IQ5 real-time PCR cycler (BioRad, USA). In total, three biological replicates were analyzed for each developmental stage for both loquat peel and flesh. The reaction mixture consisted of 4 μL cDNA in reaction buffer (15-fold diluted first-strand cDNA for all genes except for *VDE* and *LCYE* that were diluted 5-fold), 0.5 μL of each primer (10 pmol/μL) and 5 μL SensiFAST™ SYBR® & Fluorescein mix 2× (Bioline). The total reaction volume was 10 μL. The initial denaturation step was at 95 °C for 5 min, followed by 40 cycles of amplification [95 °C for 30 s, annealing temperature (Ta °C) for 30 s, and 72 °C for 30 s] and

a final elongation stage at 72 °C for 5 min. Gene amplification cycle was followed by a melting curve run, carrying out 61 cycles with 0.5 °C increment from 65 to 95 °C. The annealing temperature of previously published primers for loquat carotenoid biosynthetic genes (58 to 65 °C) is shown in Additional file 1: Table S1. Loquat's *actin* gene was used as a housekeeping reference gene (*EjACT*).

Statistical analyses

Statistical analyses were carried out by comparing the averages of each developmental stage based on the analysis of variance (one way ANOVA) according to Duncan's multiple way test with a significance level of 5% ($P \leq 0{,}05$), using the SPSS v.17.0 statistical analysis software package.

The relative quantification and statistical analysis of gene expression levels was performed with the REST-XL software, using the pairwise fixed reallocation randomization test [22]. Gene expression levels were normalized against the *EjACT* housekeeping reference gene; the initial developmental stage (S1) both for peel and flesh tissue was used for calibration.

Results and discussion

Qualitative attributes

Fruit weight, length and width ranged between 25.3–59.1 g, 48.8–57.0 mm and 34.4–45.0 mm, respectively (Additional file 1: Table S2). Maximum fruit size and weight was recorded at stage S5, which coincides with the optimum maturity stage for harvest.

Colour parameters in the flesh ranged between 52.12–74.32 for L*, −11.51-10.67 for a* and 30.75–36.67 for b*. The corresponding values for L^*, a^*, b^* parameters in the peel were 45.65–64.99, −17.66-12.42 and 28.20–49.19 (Additional file 1: Table S3). Previous study in loquat cultivars indicated that the a*/b* chroma ratio receive negative values in immature fruits, around zero for pale yellow-colored fruits, and positive values for orange-colored loquat fruits; thus higher ratio can be linked with higher carotenoid accumulation [1]. In our study, the a*/b* ratio in the flesh received negative values at stages S1-S3, implying the green colour, the S4 value indicates the colour break and the S5 and S6 had the higher values. Similarly, the a*/b* ratio was lower in the peel during the first three early stages (−0.60 to −0.20), close to zero in the breaker stage, while it received positive values during the last maturation stages (0.24–0.29) (Additional file 1: Table S3). Overall, the a*/b* ratio can be linked with total carotenoid accumulation and its transient increase with the progress of on-tree development, in accordance with previous studies [1, 16].

Carotenoid and chlorophyll contents

Initially, a rapid colorimetric assay was employed to screen carotenoid and chlorophyll contents. Total carotenoids varied between 0.8–6.1 and 0.1–5.9 mg 100 g $^{-1}$ FW β-carotene equivalents in the peel and in the flesh, respectively (data not shown). The highest concentrations in Chl a and Chl b were found in the peel tissue at the immature green stage (18.0 and 12.1 mg 100 g $^{-1}$ FW, respectively), while their contents were substantially lower in the flesh (0.6 and 0.5 mg 100 g^{-1} FW at S1 stage, respectively) and degraded thereafter with the progress of on-tree ripening. During ripening of fleshy fruits chloroplasts turn into chromoplasts; this process encompass a transient increase of carotenoids and degradation of chlorophylls [23]. Chl a is a blue-green coloured pigment and is less stable than the yellow-green Chl b.

Identification of carotenoids in loquat fruit using LC-MS

Carotenoids in the peel and in the flesh were identified and quantified (Table 1, Additional file 1: Figure S1). Thirty-two carotenoids were detected by HPLC-DAD and LC-MS techniques. Peak identification was based on their relative retention time values, their UV-Vis spectra, their mass spectra, information from the literature and comparison with authentic standards when possible. Table 1 summarizes the identification data for each carotenoid, including chromatographic and spectroscopic values.

All-*trans*-neoxanthin (peak 1) showed a characteristic UV-visible spectrum. The molecular mass of neoxanthin was confirmed by the protonated molecule at *m/z* 601 and by consecutive losses of three hydroxyl groups from the protonated molecule, at *m/z* 583, 565 and 547, verified by MS/MS. The UV-visible absorption spectrum of neochrome (peak 2) showed λmax at 397, 420 and 448 nm with high spectral fine structure (%III/II 90); these values are in agreement with previous studies in loquat [9]. All-trans-violaxanthin (peak 3) was identified by comparison with the authentic standard. The protonated molecule at *m/z* 601, and the fragments at *m/z* 583 and 565, due to the losses of hydroxyl groups and at *m/z* 221, all formed from 601 at both MS/MS and in-source fragmentations. Peak 8 was tentatively identified as *cis*-violaxanthin with a mass spectrum, lower λmax and spectral fine structure values similar to those of peak 3. β-diepoxy cryptoxanthin, peak 7 and 32 (only in flesh in S5 and S6)] showed the $[M + H]^+$ at *m/z* 585. The second order MS experiments revealed a fragment at *m/z* 567 due to the loss of water and the ions at *m/z* 221 and at *m/z* 205 characteristic of the epoxide group with one ion located in a ring with a hydroxyl group and another one in an unsubstituted ring respectively. All *trans*-lutein was identified by comparison with an authentic standard.

Table 1 UV/vis spectra and characteristic ions of carotenoids from six maturation stages of loquat fruits, obtained by HPLC-PDA-MS

Peak	Carotenoid	t_R (min)	λ_{max} (nm)	%III/II	%Ab/II	[M + H] + m/z	HPLC/APCI (+)-MS^n experiment *m/z* (% base peak)	Exact mass	Score	Error (ppm)	Molecular formula
1	All-*trans*-neoxanthin	14.1	412,436,462	70	0	601 (40), 583 (75), 565 (100)	MS^2 [601]: 583 (100), 565 (47), 547 (9), 509 (6),491 (5), 221 (41) MS^3 [601˃583]: 565 (48), 547 (14)	600.4188	91.88	-2.69	$C_{40}H_{56}O_4$
2	All-*trans*-neochrome	14.6	397,420,448	90	0	601 (42), 583 (100), 565 (43)	MS^2 [601]: 583 (100), 565 (57), MS^3 [601˃583]:221 (62)	600.4178	96.70	-0.89	$C_{40}H_{56}O_4$
3	All-*trans*-violaxanthin	14.8	414,438,470	75	0	601 (85), 583 (100), 565 (20)	MS^2 [601]: 583 (100), 565 (12), 509 (5), 221 (24)	600.4178			$C_{40}H_{56}O_4$
4	Not identified	16.0	466	0	0	455 (100)	MS^2 [455]: 437 (80), 399 (34)	455.3324	93.60	-2.61	$C_{33}H_{43}O$
5	Not identified	16.2	396,420,448	75	0	601 (60)	MS^2 [601]: 583 (95), 221 (100				
6	Not identified	16.4	Not detected	–	–	601 (64)	MS^2 [601]: 583 (90), 565 (40), 491 (9), 221 (100) MS^3 [601˃583]:565 (100),221 (60)				
7	β- Diepoxy-cryptoxanthin	16.7	412, 436,466	72	0	585 (100)	MS^2 [585]: 567 (45), 549 (80), 493 (37), 221 (100), 205 (10)	584.4184	95.60	-2.52	$C_{40}H_{56}O_3$
8	*Cis*-violaxanthin	17.3	324,410, 434,464	60	8	601 (81), 487 (100)	MS^2 [601]: 583 (100), 565 (42), 509 (6), 491 (23), 221 (51) MS^2 [487]: 469 (100)				
9	Not identified	17.8	378,400,424	100	0	601 (100), 351 (98)	MS^2 [601]: 583 (100), 565 (13), 509 (15), 491 (14),393 (40),221 (41) MS^2 [351]: 333 (16)				
10	All-*trans*-lutein	18.2	420,444,472	48	0	569 (5), 551 (100)	MS^2[551]: 533 (51), 495 (24), 477 (35)	568.427	99.30	-0.226	$C_{40}H_{56}O_2$
11	Not identified	18.5	444	0	0	454 (100)	MS^2[454]: 436 (7), 393 (100)				
12	Not identified	18.9	448	0	0	473 (100),539 (80)	MS^2[539]: 521 (100)				
13	Not identified	19.0	454	0	0	473 (100), 454 (14)	MS^2 [473]: 455 (43), 205 (100) MS^2 [454]: 436 (31), 393 (36)				
14	Not identified	19.3	423,438,474	60		551 (100)	MS^2 [551]: 533 (67), 477 (55)				
15	Not identified	19.4	448	0	0	439 (93), 403 (100)	MS^2 [439]:403 (14)				
16	Not identified	19.6	434	0	0	391 (100)	MS^2 [391]:373 (14)				
17	5,6-Epoxy-β-cryptoxanthin	19.8	416,438,466	34	0	569 (17),551 (54)	MS^2 [551]: 533 (20), 205 (21)	568.428	75.56	-5.07	$C_{40}H_{56}O_2$
18	5′,6′-Epoxy-β-cryptoxanthin	19.9	419,441,470	52	0	569 (30), 551 (100)	MS^2 [551]: 533 (100), 577 (14), 459 (30), 221 (13)	568.428	89.42	-2.11	$C_{40}H_{56}O_2$
19	*Cis*-lutein	20	326,412,436,462	52	20	569 (18), 551 (100)	MS^2 [551]: 533 (100), 221 (11)	568.428	77.7	3.57	$C_{40}H_{56}O_2$
32	β- Diepoxy-cryptoxanthin	20.2	416,440, 470	87	0	585 (100)	MS^2 [583]: 567 (49), 549 (10) 221 (31), 205 (29)				
20	Not identified	20.4				446 (100) 417 (90)	MS^2 [417]:399 (100) MS^2 [446]:219 (100)				

Table 1 UV/vis spectra and characteristic ions of carotenoids from six maturation stages of loquat fruits, obtained by HPLC-PDA-MS *(Continued)*

21	β-Apo-8′-carotenal (IS)	21.0	450	0	0	417 (100)	MS^2 [417]:399 (25), 389 (32), 361 (39), 325 (100), 293 (97), 157 (88), 119 (16)	416.3079	98.23	-2.15	$C_{30}H_{40}O$
22	Citranaxanthin	21.6	470	0	0	457 (100)	MS^2 [457]:439 (90), 399 (49)	455.3324	97.23	-1.25	$C_{33}H_{44}O$
23	Not identified	22.4	470			696 (100)	MS^2 [696]: 534 (34), 516 (100)				
24	Not identified	22.8	420,444,470	31	0	537 (58)	MS^2 [537]: 467 (16), 444 (51), 365 (100)				
25	Not identified	23.3	Not detected			537 (28),430 (100)	MS^2 [537]:444 (100),481 (24) 413 (90)				
26	All-*trans*-β-cryptoxanthin	24.0	420, 448,472	24	0	553 (100)	MS^2 [553]:535 (100), 497 (41),461 (10)				
27	Not identified	24.5				664 (100)	MS^2 [664]: 551 (100), 496 (55)				
28	Not identified	24.9	453, 479	0	0	551 (100)	MS^2 [551]: 534 (15), 361 (100)				
29	Phytoene + not identified 14	28.0				545 (95), 553 (100)	MS^2 [545]:489 (10),395 (100) MS^2 [553]:535 (12)				
30	5,8-epoxy-β-carotene	28.4	405, 424, 450	26	0	553 (100)	MS^2 [553]:535 (55),461 (35), 221 (64), 205 (17)				
31	All-*trans*-β-carotene	36.5	424, 446,470	18	0	537	MS^2 [537]:444 (100)				

All *trans*-lutein (peak 10) and *cis*-lutein (peak 19) showed characteristic UV-visible spectra, with a hypsochromic shift of 8 nm for the *cis* isomer. The identification of both lutein isomers was confirmed by their mass spectra with the protonated molecule at *m/z* 569 and fragments at *m/z* 551 and *m/z* 533 due to the loss of one and two hydroxyl group respectively. The MS/MS showed, in addition, the presence of fragments at *m/z* 477 resulting from the loss of toluene ([M + H-92] $^{+}$) from the polyene chain. In APCI-MS, the fragment with *m/z* 551 presented a higher intensity than the protonated molecular ion (*m/z* 569). Peaks 17 and 18 were identified as mono-epoxides of β-cryptoxanthin considering their UV-vis and MS characteristics by the comparison with literature data [9, 24]. The mass spectra of both epoxides presented the protonated molecule at *m/z* 569 and fragment ion at *m/z* 551 due to the loss of a hydroxyl group. Peak 17 was designated as 5′,6′-epoxy- β-cryptoxanthin due to the presence of the mass fragment at *m/z* 205 that is consistent with the location of one epoxide group in the unsubstituted ring whilst peak 18 showed the mass fragment at *m/z* 221, indicating that the epoxide groups were in a ring with a hydroxyl group. β-Apo-8′-carotenal was identified as peak 21 (internal standard). The mass spectra presented the protonated molecule at *m/z* 417. Ions of *m/z* 399 and 389 were detected corresponding to the loss of water and carbon monoxide respectively. Elimination of toluene from the protonated molecule was observed at *m/z* 325. The use of an internal standard was recommended to estimate the losses during the extraction process. Saponification with potassium hydroxide has been an integral part of carotenoid analyses. Kimura et al. [25] showed that β-apo-8′-carotenal was completely transformed to citranaxanthin (peak 22), apparently by aldol condensation with acetone. The conversion percentage from β-apo-8′-carotenal to citranaxanthin was 98% and their sum was considered for quantification. The identification of citranaxanthin was confirmed on the basis of its protonated molecule at *m/z* 457 [26] and its characteristic UV-vis spectrum [27]. Due to the presence of the same chromophore, β-cryptoxanthin (peak 26) and β-carotene have similar UV-visible spectra. As expected, the protonated molecule was detected at *m/z* 553 and the MS/MS revealed the presence of fragment ions at *m/z* 551 and 461 corresponding to the losses of the hydroxyl group and toluene. 5,8-epoxy-β-carotene (peak 30) could not be identified by its UV-visible spectral characteristics. Mass spectra highlighted the protonated molecule at *m/z* 553 and the MS/MS showed the presence of fragment ions at *m/z* 551 and 461 corresponding to the losses of the hydroxyl group and toluene, respectively, and at *m/z* 221 that corresponds to the location of the epoxide group in the 3-hydroxy-β-ring. The mass spectra of beta-carotene, peak 31, showed the protonated molecule at *m/z* 537 and a fragment ion in the MS/MS at *m/z* 444, corresponding to the loss of the toluene from the polyene chain.

Carotenoid composition in loquat fruit

Results revealed great differences in carotenoid composition between peel and flesh. In particular, 32 carotenoids were found in loquat peel, while only eighteen carotenoids were identified in the flesh. Except for qualitative differences, the concentration of carotenoids was significantly higher in the peel than in the flesh. This was not the case when total carotenoids were determined spectrophotometrically, indicating the limitations of such colorimetric assays. Chromatograms also revealed that the major carotenoids in peel were *trans*-lutein and *trans*-β-carotene. The concentration of *trans*-lutein decreased with the progress of ripening from 1621.5 to 688.4 μg 100 g^{-1} FW. On the other hand, *trans*-β-carotene content increased drastically from 151.9 to 1096.9 μg 100 g^{-1} FW. The biosynthesis of some carotenoids such as *trans*-β-cryptoxanthin, 5,8-epoxy-β-carotene, β-diepoxy-cryptoxanthin and *cis*-violaxanthin has also been monitored (Fig. 3, Additional file 1: Table S4). Conversely, *trans*-neoxanthin and *trans*-neochrome decreased or remained stable with the progress of on-tree fruit development in the peel, while they were not detected in the flesh during the last developmental stages.

The carotenoid profiling of loquat flesh was found to be quite different from the peel. The most abundant carotenoid in mature fruits was *trans*-β-cryptoxanthin, followed by *trans*-β-carotene, compounds 18 and 31, and 5,8-epoxy-β-carotene (peak 30) (Table 1, Fig. 3, Additional file 1: Table S4). An increment in the concentration of all carotenoids during on-tree development except for *trans*-neoxanthin, *trans*-neochrome and *trans*-lutein was found (Fig. 3, Additional file 1: Table S4). Overall, a great effect of the developmental stage on the carotenoid composition was revealed.

All-*trans*-neochrome, all-*trans*-violaxanthin, β-diepoxy-cryptoxanthin, *cis*-violaxanthin, all-*trans*-lutein, 5,6-epoxy-β-cryptoxanthin, 5′,6′-epoxy-β-cryptoxanthin, all-*trans*-β-cryptoxanthin, phytoene and all-*trans*-β-carotene were previously identified in five loquat cultivars, originating from Brazil [9]. In their findings, they reported *trans*-β-carotene (19–55%), *trans*-β-cryptoxanthin (18–28%), 5,6:5,6 -diepoxy-β-cryptoxanthin (9–18%) and 5,6-epoxy-β-cryptoxanthin (7–10%) to be the main carotenoids. In the flesh, it was found that β-carotene and lutein were the major carotenoids with neoxanthin, violaxanthin, luteoxanthin, 9-*cis*-violaxanthin, phytoene, phytofluene and ζ–carotene also present.

The carotenoid quantification in our study proved that the peel had higher carotenoid concentrations than the

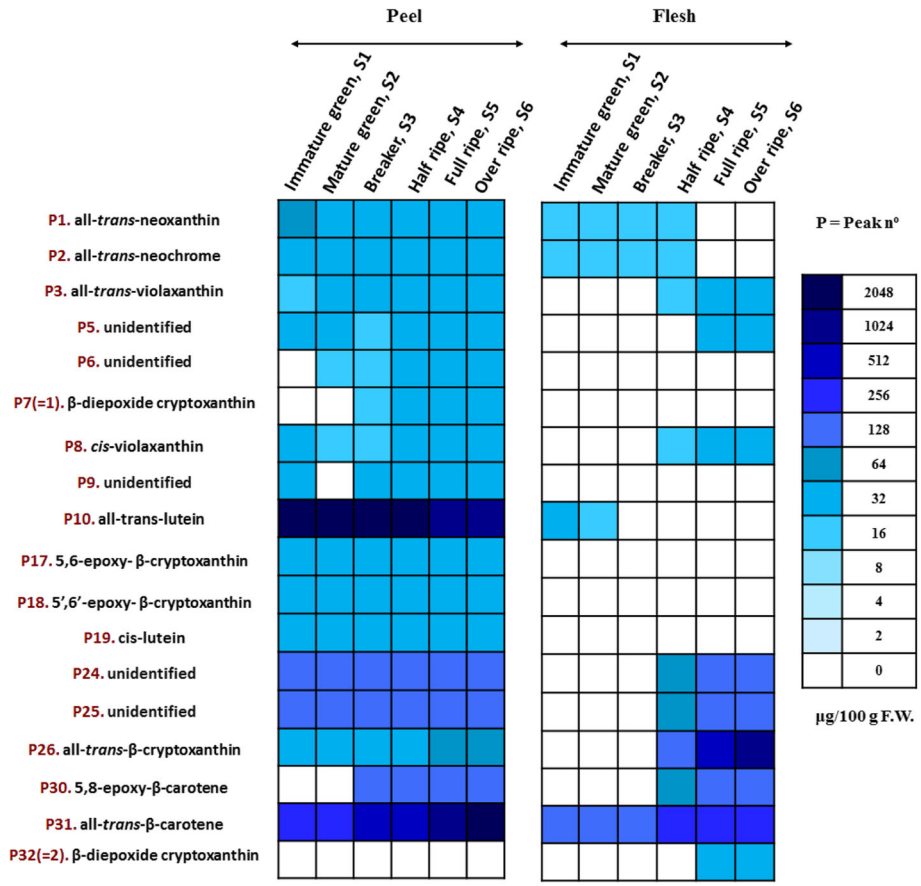

Fig. 3 Heat map of the quantification of the identified carotenoids (Table 1) in the peel and in the flesh of loquat fruit (cv. 'Obusa') during six on-tree developmental stages ($n = 3$). Results are expressed as μg 100 g^{-1} fresh weight (F.W.). A scale of colour intensity is presented as a *legend*. Actual quantification values of the identified carotenoids are shown in Additional file 1: Table S4

flesh, except for β-diepoxy-cryptoxanthin which was found from the 3rd until the 6th maturity stage in the peel, ranging from 15.0 ± 0.8 to 21.2 ± 1.7 μg 100 g^{-1} FW, as well as in flesh during the last maturity stages (S5 and S6) at 17.6 ± 1.2 and 21.4 ± 1.8 μg 100 g^{-1} FW, respectively (Fig. 3, Additional file 1: Table S4). *Trans*-neoxanthin and *trans*-neochrome appeared throughout all developmental stages in the peel as well as in the first 4 maturation stages in the flesh. On the other hand, *trans*-violaxanthin increased in the peel with the progress of on-tree ripening (15.6 ± 2.5 to 25.2 ± 4.6 μg 100 g^{-1} FW), while it was detected in the flesh during the last stages (S4-S6) with progressive increase (from 12.5 ± 0.2 to 21.5 ± 1.6 μg 100 g^{-1} FW). Similar findings and trend were observed for *cis*-violaxanthin which ranged from 16.2 ± 2.5 to 23.2 ± 3.0 μg 100 g^{-1} FW in the peel, being detectable in the flesh from S4 stage onwards (12.3 ± 0.1 to 19.1 ± 1.5 μg 100 g^{-1} FW). 5′,6′-Epoxy-β-cryptoxanthin and *cis*-lutein were detected exclusively in the peel throughout all developmental stages (Fig. 3). Intriguingly, *trans*-lutein in the peel registered the highest contents during the initial developmental stages and went descending thereafter, meanwhile it was found in detectable amounts in the flesh only at stages 1 and 2, yet substantially lower compared to the peel. Citranaxanthin and phytoene have also been identified, although they were not quantified (Table 1).

Gene expression profiles

With the aim to shed light on the carotenoid biosynthetic pathway in loquat fruit, the expression profile of thirteen known genes of the carotenoid pathway was analyzed, showing differential expression patterns in the peel and the flesh tissue (Fig. 4, Additional file 1: Tables S5–S6 and Figures S2–S3). For gene expression analyses, each tissue was examined individually, considering S1 stage as the calibrator of the tissue tested (peel or flesh).

Middle and downstream genes of the carotenoid biosynthetic pathway (*CYCB, LCYE, BCH, ECH* and *VDE*) showed clear differentiation in their expression levels among S2-S6 developmental stages compared with the remaining genes. In both peel and flesh, *PSY1*, *ZDS*,

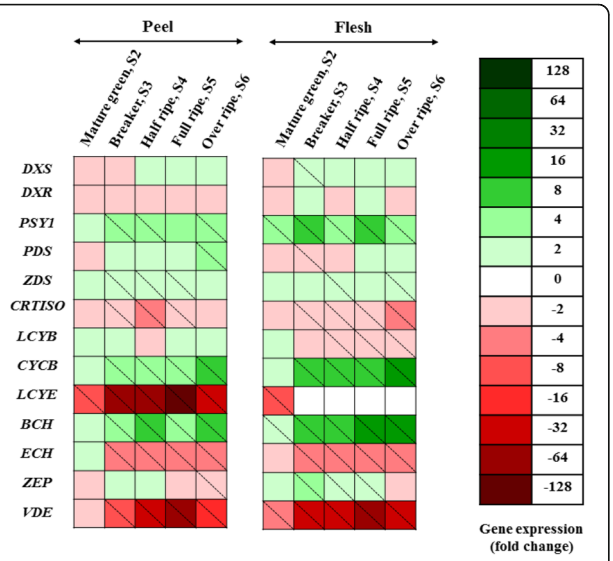

Fig. 4 Heat map of the relative expression levels of carotenoid biosynthesis genes (*DXS, DXR, PSY1, PDS, ZDS, CRTISO, LCYB, CYCB, LCYE, BCH, ECH, ZEP* and *VDE*) in loquat fruit (cv. 'Obusa'), both in the peel and in the flesh, during five on-tree developmental stages (S2-S6) ($n = 3$). Relative mRNA abundance was evaluated by real-time RT-PCR using three biological repeats. Up-regulation is indicated in *green*; down-regulation is indicated in *red*. A *diagonal line in a box* indicates a statistically significant value ($P \leq 0.05$). A scale of colour intensity is presented as a *legend*. The first developmental stage (S1) both for the peel and the flesh was used for calibrating gene exeression values. Actual relative expression levels are shown in Additional file 1: Tables S5-S6 and Figs. S2-S3

CYCB, and *BCH* were significantly up-regulated in most of the developmental stages compared with the S1 stage respectively, whereas *CRTISO*, *LCYE*, *ECH* and *VDE* were generally down-regulated. *PDS* was significantly up-regulated at the S6 stage in the peel, while it was significantly suppressed at the S3 stage in the flesh tissue. *ZDS* presented an increase in expression levels between the S3 and S5 stage in peel, though the increase was at the S4 and S6 stage in flesh tissue. Non statistically significant changes in expression levels of *DXS, DXR* (both involved in MEP pathway) and *LCYB* were monitored in the peel in comparison with the S1 stage; in flesh *DXR* followed the same trend whereas, *DXS* presented an accumulation at the S3 and *LCYB* transcript levels decreased gradually through stages S4-S6 which could account for the low or non-detectable *trans*-lutein findings in the flesh (Figs. 1, 3 and 4). *ZEP* expression levels presented highest suppression at the S6 stage in peel, contrarily to flesh profile where it was up-regulated at the S3-S5 stages.

BCH and *CYCB* transcript levels were substantially up-regulated with the progress of on-tree fruit development both in the peel and in the flesh, registering the highest values at the last stage for both tissues (Fig. 4, Additional file 1: Tables S5, S6). Notably in flesh tissue, a 8.1- and 11.0- fold increase of *CYCB* and *BCH* transcripts during the last stage compared to the initial stage was recorded, respectively. *PSY1* presented statistically significant increases at the S3, S4 and S6 stages of the peel and throughout all stages in the flesh (S2-S6); the most prominent gene regulation in the flesh was found at the S3 and S5 stages (Additional file 1: Table S6).

Contrarily, *CRTISO, LCYE, ECH and VDE* demonstrated overall a down-regulation expression pattern in both peel and flesh tissue. *CRTISO* was down-regulated at stages S3-S5 in peel; a similar trend was monitored in the flesh (S3-S6 stages). *ECH* had similar expression pattern (suppression) for both peel and flesh from stage S3 onwards. *LCYE* was down-regulated in peel throughout the five developmental stages; the most abundant decrease (128 fold change) was registered at the S5 stage. Interestingly, in flesh the decrease was registered at the S2 stage and remained undetected thereafter. *VDE* expression levels were repressed throughout S3 to S6 stages in peel and throughout all developmental stages in flesh. In both peel and flesh, *VDE* presented a substantial suppression at the S5 stages (36.8 and 49.5 fold change, respectively) compared with S1 stage.

Notably, *PSY1*, known to catalyse the first step in the carotenoid formation [14], expression levels depicted a general up-regulation with the progress of on-tree fruit development both in the peel and in the flesh; however the highest transcript values in flesh tissue were monitored at S3 and S5 stages, not concomitant with total carotenoid accumulation. In another fleshy fruit (apple), Ampomah-Dwamena et al. [28] also postulated that *PSY1* expression levels had no direct correlation with carotenoid content in different genotypes. On the other hand, *PDS*, an upstream pathway gene, presented statistically significantly higher transcript levels only in the peel at the last developmental stage (S6); *PDS* expression levels have been correlated with high- and low- carotenoid content apple cultivars [28].

Trans-lutein presented appreciably high accumulation in the first four stages in the peel with a reduction at ripe and over-ripe stages, while detectable amounts in the flesh were registered only during the initial developmental stages, as chloroplasts began to develop into chromoplasts. This decrease can be attributed to the fact that (1) *LCYE* was markedly down-regulated throughout the developmental stages in both peel and flesh compared to corresponding per tissue immature stage (notably not detectable transcripts during S3-S6 of flesh was monitored), (2) *LCYB* was down-regulated over the last developmental stages in flesh (S4-S6) and (3) *ECH* mRNA expression was generally down-regulated both in the peel and in the flesh (S3-S6). Fu et al. [10] noted that lutein is showing a transient decrease in the flesh of loquat cultivars with the progress of on-tree fruit

development, whereas there is no connection with *BCH* expression which appears to be up-regulated especially in the red-fleshed cultivar 'Luoyangqing'. An up-regulation of *BCH* gene expression was also monitored in our study in a similar flesh-type loquat cultivar. Ampomah-Dwamena et al. [28] showed a close correlation between *LCYE* expression and carotenoid content in apple fruit skin. This was not the case with the flesh; suggesting that down-regulation of *LCYE* is consistent with lower *trans*-lutein concentrations in the flesh.

Contrarily, the high *BCH* expression values registered in the flesh at the last two stages can be linked with the transient carotenoid accumulation of *trans*-β-cryptoxanthin in these stages, concomitant with higher mRNA expression of *CYCB* and non-detectable *LCYE* transcripts (Stages S3–S6). These findings are in accordance with Fu et al. [10], where the *BCH* values for the red-fleshed cultivar 'Luoyangqing' showed a transient increase at the breaker stage. In addition, *CYCB* expression levels were also higher after the S4 stage. Kato et al. [11] stated that the decrease of *LCYE* gene expression in Citrus fruits is related with higher contents of β-carotene as the ε, β-branch of the carotenoid pathway shifts to the β, β-branch during transition from immature to mature stage. Zhao et al. [16] found that *BCH* is responsible for high β-cryptoxanthin content in persimmon fruit, in accordance with findings in other loquat cultivars [29]. The latter study suggests a direct link between the synthesis and accumulation of β-cryptoxanthin and the abundant expression of *BCH*. The transient increase of *trans*-β-carotene towards the S6 peel stage can also be linked with the up-regulation of *CYCB* and down-regulation of *LCYE,* as elsewhere described [10]. Zhang et al. [29] also links the higher β-carotene level in loquat peel with the abundant increase of *PSY1*, as well as *CYCB* and *BCH* mRNA expression levels.

VDE expression which leads to violaxanthin biosynthesis is significantly suppressed in almost all stages, both in the flesh and in the peel compared with the calibrator (S1 stage) (thus expecting very little violaxanthin); in the case of *ZEP*, which converts violaxanthin back to precursor molecules such as zeaxanthin, the main trend is that it is induced in several stages in the flesh. This is in accordance with metabolite levels, as both *cis*- and *trans*-violaxanthin are at appreciable low concentrations and/or non-detectable during several stages in the flesh (Figs. 3, 4; Additional file 1: Table S4).

Conclusions

The carotenoid profile of 'Obusa' fruits, an orange-fleshed loquat cultivar, was elucidated during distinct on-tree developmental stages. Results indicated that carotenoid composition was greatly affected during fruit development, revealing evident differentiations between flesh and peel tissue. The major carotenoids were *trans*-lutein and *trans*-β-carotene in the peel, and *trans*-β-cryptoxanthin, *trans*-β-carotene, and 5,8-epoxy-β-carotene in the flesh. To the best of our knowledge, the presence of *cis*-lutein, citranaxanthin and 5,8-epoxy-β-carotene has not been reported previously in loquat, but only in other fruits of tropical origin [30, 31]. Furthermore, a link was attempted to be established between gene up- or down-regulation during the developmental stages of the loquat fruit, and how their expression affects carotenoid content. Elevated content of *trans*-β-carotene both in the flesh and in the peel with the progress of on-tree fruit development can be linked with the up-regulation of *CYCB,* a main carotenoid biosynthetic gene. Notably, the non-detectable amounts of *trans*-lutein in the flesh during the S3-S6 stages can be linked with the significant suppression of *LCYB* and *LCYE* expression levels during these stages. Transcripts levels of the latter gene were also significantly reduced throughout all developmental stages in the peel compared with the immature stage.

Additional file

Additional file 1: Figures S1-S3 and Tables S1-S6 illustrate extra information cited in the text. (ZIP 182 kb)

Abbreviations

ABA: Abscisic acid; *BCH*: *β-carotene hydroxylase*; Chl a: Chlorophyll-a; Chl b: Chlorophyll-b; *CRTISO*: *Carotene isomerase*; *CYCB*: *Chromoplast-specific lycopene β-cyclase*; DMAPP: Dimethylallyl pyrophosphate; *DXR*: *DXP reductoisomerase*; *DXS*: *1-deoxy-D-xylulose 5-phosphate-synthase*; *ECH*: *ε-carotene hydroxylase*; *EjACT*: *Eriobotrya japonica Actin*; FW: Fresh weight; GAP: D-glyceraldehyde 3-phosphate; GGPP: Geranylgeranyl diphosphate; *GGPS*: *Geranylgeranyl diphosphate synthase*; HMBPP: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; *IDI*: *Isopentenyl pyrophosphate isomerase*; *IDS*: *Isopentenyl pyrophosphate synthase*; IPP: Isopentenyl pyrophosphate; *LCYB*: *Lycopene β-cyclase*; *LCYE*: *Lycopene ε-cyclase*; *NCED*: *9-cis-epoxycarotenoid dioxygenase*; *NSY*: *Neoxanthin synthase*; *PDS*: *Phytoene desaturase*; *PSY1*: *Phytoene synthase*; *VDE*: *Violaxanthin de-epoxidase*; *ZDS*: *f-carotene desaturase*; *ZEP*: *Zeaxanthin epoxidase*; *ZISO*: *ζ-carotene isomerase*

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Availability of data and materials

All data supporting the findings is contained in the manuscript and its supplementary files.

Authors' contributions

GAM conceived the project and designed the experiments. VF, MH and ECG undertook the molecular experiments. FB, AM, and HMDM undertook the analytical experiments. MH, ECG, VG, VF, AM and HMDM were involved in the data analysis. MH, ECG, AM, HMDM, VG, VF, FB and GAM wrote the paper. All authors read and approved the final manuscript.

Competing interests
Loquat fruits were harvested from a commercial orchard owned by MH. Authors declare non-financial competing interests that may cause them embarrassment if they were to become public after the publication of the manuscript.

Consent for publication
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Ethics approval and consent to participate
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Research article

Dissection of the incidence and severity of purple spot physiological disorder in loquat fruit through a physiological and molecular approach

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ABSTRACT

Loquat (*Eriobotrya japonica*) fruit marketability is affected by the incidence and severity of purple spot (PS), a pre-harvest physiological disorder showing an evident skin discoloration with depressed surface. Despite its impact in limiting the cultivation and economic potential of loquat, the etiology of this disorder is still poorly understood. To this end, our study aimed to investigate and disclose possible mechanisms underlying PS development. The intensity and severity of PS in three loquat cultivars ('Morphitiki', 'Karantoki' and 'Obusa') was phenotypically monitored during successive on-tree fruit developmental stages. 'Obusa' fruits harvested at commercial maturity stage showed the highest incidence of purple spot (58.6%), while 'Morphitiki' fruits did not show any symptoms. 'Karantoki' fruits demonstrated an intermediate response, with 31.3% of the fruit being affected. Thereafter, fruits with 30–50% PS severity were selected and used for further analysis; peel tissue was removed from both symptomatic and asymptomatic tissue of the same fruit for all examined cultivars. 'Karantoki' fruit with PS were characterized by the highest accumulation of total soluble sugars, sucrose, glucose and fructose contents, while the concentration of these primary metabolites was the lowest in asymptomatic fruit of 'Obusa', exception made for the sucrose. The incidence of PS was also transcriptionally investigated by assessing the mRNA profile of important genes involved in polyphenolic (*PAL1*, *PAL2* and *PPO1*) and carbohydrate (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) pathway. The enhanced expression levels of *CWI3* and *VI* genes in symptomatic fruit of the highly susceptible cultivar 'Obusa' highlight a cultivar-specific type of response. Notably, *SuSy* registered significantly suppressed levels in symptomatic tissue of both 'Obusa' and 'Karantoki'. To what extent *PPO is* associated with PS incidence and whether the etiology of the disorder can be assigned to an oxidative process triggered and coordinated by its action need to be further elucidated. The aforementioned genes are suggested to be further examined as potential markers towards a more sophisticated and informed characterization of purple spot detection in loquat fruit.

1. Introduction

Loquat (*Eriobotrya japonica* Lindl) is a subtropical evergreen tree crop that nowadays is receiving a growing and considerable interest from consumers due to its intrinsic properties (Gisbert et al., 2009). Loquat fruit ripen during early spring (Badenes et al., 2013), when other fresh fruit commodities are not yet available to consumers (Hadjipieri et al., 2017). The marketability of loquat is however severely affected by the development of purple spot (PS), a preharvest physiological disorder leading to skin discoloration. This disorder mainly affects the fruit side exposed to direct sunshine, heavily compromising general fruit appearance (Gariglio et al., 2002; Hadjipieri et al., 2020). Gariglio et al. (2002) postulated that purple spot initiates at the level of the inner peel cells of the fruit, causing cellular dehydration between the shrunken

Abbreviations: FW, Fresh weight; PS, Purple spot; SI, Severity incidence; TSS, Total soluble sugars; Suc, Sucrose; Glu, Glucose; Fru, Fructose; SE, Standard error; ACT, Actin; PAL, Phenylalanine ammonia lyase; PPO, Polyphenol oxidase; CWI, Cell wall invertase; SPS, Sucrose phosphate synthase; NI, Neutral invertase; SuSy, Sucrose synthase; HXK, Hexokinase; FRK, Fructokinase; VI, Vacuolar invertase.

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cytoplasm and the cell wall.

The severity of PS symptoms may substantially vary from year to year, and early harvests are generally characterized by higher incidence (Gariglio et al., 2003). A correlation between PS appearance and fruit flesh sugar concentration has been also proposed, since fruit thinning, which increased the soluble sugar accumulation in on-tree fruit, led to a higher incidence of the disorder. In addition, low temperature at night and sun exposure also increased the gradient of total sugar concentrations in favor of the flesh, contributing thereafter to an enhanced incidence of PS in the peel (Gariglio et al., 2008). Overall, the higher PS incidence has been attributed to the simultaneous occurrence of a higher accumulation of sugar in the flesh, possibly due to an enhanced growth rate stimulated by fruit thinning, and environmental factors, such as low night temperatures and direct sunlight exposure. However, to what extent such factors affect PS appearance still remains unknown.

Soluble sugars, including sucrose (Suc), fructose (Fru) and glucose (Glu), are widely considered as the major source of energy useful to accomplish several metabolic processes and defense mechanisms (Li et al., 2012). Fruit growth and development relies on Suc supply, which is also generally used as a major pomological index to determine the most appropriate harvesting date in many fruits. To this end, the sucrose transportation in the fruit represents an essential component of this metabolism. Briefly, after Suc is transported into sink cells (fruit, root, etc.), it is then converted into Fru and Glu by the action of *neutral invertase* (*NI*) (Fotopoulos, 2005; Li et al., 2012) or to UPD glucose by *sucrose synthase* (*SuSy*) (Zhou et al., 2006). In turn, Glu and Fru are then phosphorylated to glucose-6-phosphate and fructose-6-phosphate by hexokinase (HXK) and fructokinase (FRK), respectively. The pathway involves Suc transport through the apoplast via Suc transporters or hydrolysis to hexoses by *CWI* or *SuSy* and subsequent uptake by monosaccharide transporters (Nguyen-Quoc and Foyer, 2001; Li et al., 2012). Special transporter proteins, located in the membrane of the vacuole, transport most of the Suc, Fru, Glu (together with other soluble sugars) that have not been metabolized in the vacuole. *Vacuolar invertase* (*VI*) converts Suc to Fru and Glu, while hexose transporters transport the converted Glu and Fru [derived from Suc by *cell-wall invertase* (*CWI*)] into the parenchyma (Li et al., 2012).

Loquat fruit belong to the Rosaceae family, in which a similar peel discoloration has been documented in other species, such as apple and pear. In these species, the phenomenon is known as superficial scald, a postharvest disorder attributed to a chilling injury event that is restricted mainly in the underlying cell layer (Lurie and Watkins, 2012). The development of this disorder in apple is due to the metabolic reaction of the oxidation of chlorogenic acid (stored in the vacuole) by the action of the polyphenol oxidase (accumulated in the plastid) as reaction of the loss of internal membrane compartmentalization (Busatto et al., 2014, 2018), and a similar mechanism has been also suggested for the superficial scald disorder in pear (Giné-Bordonaba et al., 2020).

The aim of the current study was to to dissect the potential mechanisms regulating the development of PS in loquat fruit through the investigation of sugar metabolism along with expression levels of key genes involved in carbohydrate and polyphenolic pathways.

2. Materials and methods

2.1. Fruit material and sample preparation

Trees with contiguous vigor and canopy uniformity of three loquat cultivars ('Morphitiki', 'Karantoki', 'Obusa') grown in a commercial orchard in Cyprus (Episkopi, Lemesos) were employed as experimental material in the current study. The orchard was covered, with white 17-mesh net for protection against extreme weather conditions (hail, strong winds, sunshine) and bird control. Upon fruit set and immediately after fruit thinning, length and width from ten fruits per cultivar were measured at 7-day intervals for twelve weeks with the use of an electronic caliber (IS11112, Insize).

'Karantoki' and 'Obusa' fruits with PS severity of 30–50% at fully-ripe stage were selected for carbohydrate determination and gene expression analysis. Sugars and total RNA were extracted from peel tissue (both asymptomatic and symptomatic parts from the same fruit) of three biological replications per cultivar. In the case of 'Morphitiki' fruits that was symptom free of PS disorder, only asymptomatic peel tissue was excised. All peel tissues were immediately frozen in liquid nitrogen and kept at – 80 °C until further analyses.

2.2. Severity index of purple spot

Loquat is characterized by scalar on-tree ripening. The incidence and severity of PS was monitored in the fruits harvested during the 'first hand', when such symptoms are more evident. Fruit were harvested based on size and background colour and subsequently were segregated into five distinct groups according to the PS severity defined as follows: SI-1: asymptomatic or with slight symptoms (up to 10% of fruit total surface), SI-2: 10–30% of affected surface, SI-3: 30–50% of affected surface, SI-4:50–70% and SI-5: 70% or higher affected surface. Subsequently, severity index was calculated as follows with NF standing for number of fruits.

$$\text{Severity Index} = [\text{NF(SI}-5\text{x}2) + \text{NF(SI}-4\text{x}1) + \text{NF(SI}-3\text{x}0.5) + \text{NF(SI}-2\text{x}0.25) + \text{NF(SI}-1\text{x}0)] \,//\, \text{total fruits}$$

2.3. Total soluble sugars (TSS), sucrose, glucose and fructose determination

Peel sugars (TSS, Suc, Glu, Fru) were extracted using the method described by Jin et al. (2007) with slight modifications. The sample (0.16 g of tissue) was extracted with 5 mL of ethanol (80%, v/v) and vortexed. The extracts were then incubated at 85 °C for 5 min and subsequently centrifuged (21191 ×*g* for 10 min at 4 °C). The supernatant was removed and the pellet was re-extracted three additional times in 80% (v/v) ethanol as above. The supernatant from each extraction was pooled, vacuum-dried at 45 °C, re-solubilized in 15 ml of hot distilled water and vortexed. After 45 min incubation in an ultrasonic bath (Ultrasonic Cleaner, Raypa, UCI-150) at 40 °C, crude extract supernatants were decanted and held at −20 °C until further analysis.

TSS were analysed by reacting 0.1 mL of the alcoholic extract with 3 mL fleshly prepared anthrone (150 mg anthrone and 100 mL 72% v/v H_2SO_4) in boiling water bath (40 °C) for 10 min and then cooled. TSS was then calculated spectrophotometrically at 625 nm (TECAN, Infinite 200® PRO) using sucrose as a standard (Jin et al., 2007).

For sucrose, 0.1 mL extract was heated with 0.1 mL 5.4 M KOH for 10 min at 95 °C. Following the cooling of the reactive product, 3 mL of freshly prepared anthrone reagent were added and the mixture was again heated at 95 °C for 5 min, cooled, and sucrose content was determined based on absorbance values at 620 nm (Jin et al., 2007).

For glucose, 1 mL of the alcoholic extract was heated with 5 mL *o*-toluidine reagent (15 mL *o*-toluidine and 0.5 g thiourea reaching a volume of 250 mL with the use of glacial acetic acid) for 15 min at 95 °C. Absorbance was measured at 630 nm and glucose content was determined as elsewhere described (Jin et al., 2007).

For fructose, 2 mL of the extract were added to 1 mL of resorcinol reagent (0.6 g resorcinol and 0.15 g thiourea reaching 100 mL glacial acetic acid) and 7 mL HCL/water (5/1, v/v). From the working standard solution, six volumes (0, 0.2, 0.4, 0.6, 0.8 and 1 mL) were placed into different tubes and reached the volume of 2 mL with addition of distilled water. Thereafter, 1 mL of resorcinol reagent and 7 mL of HCL were added and all tubes were heated in water bath at 80 °C for 10 min. Once the mixture cooled down, absorbance values at 520 nm were monitored and transformed to fructose content as elsewhere described (Edewor-Kuponiyi, 2013).

All chemical reagents (thiourea, o-toluidine, resorcinol and

anthrone) and standard compounds of sucrose, glucose and fructose were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.4. Gene expression analysis

The expression levels were carried out selecting important key genes involved in the polyphenol (*PAL1*, *PAL2* and *PPO1*) and carbohydrate (*CWI2*, *CWI3*, *SPS1*, *SPS2*, *NI2*, *NI3*, *SuSy*, *HXK*, *FRK* and *VI*) pathway as illustrated in Fig. 1. Extraction of RNA was carried out based on a modified cetyltrimethylammonium bromide (CTAB) protocol originally developed by Gambino et al. (2008). RNA integrity was spectrophotometrically assessed (Nanodrop 1000 Spectrophotometer, Thermo Scientific) and visually inspected with gel electrophoresis. Samples were then treated with RNase-free DNase (Cat. No. NU01a, HT Biotechnology LTD, England) to remove total gDNA, as elsewhere described (Georgiadou et al., 2016).

PrimeScript™ RT reagent kit (Takara Bio, Japan) was used to reverse transcribed total RNA (0.5 μg), according to the manufacturer's protocol (Takara Bio, Japan). Quantitative real-time RT-qPCR was carried out with BioRad IQ5 real-time PCR cycler (BioRad, USA). In total, three biological replicates were performed for cvs. 'Karantoki' and 'Obusa', on both symptomatic (30–50%) and asymptomatic peel tissue of at fully-ripe stage. For cv. 'Morphitiki' only asymptomatic peel tissue was assessed. The reaction mixture of 10 μL consisted of 4 μL cDNA in reaction buffer consisting of 0.5 μL of each primer (10 pmol/μL), 5 μL SensiFAST™ SYBR® and Fluorescein mix 2x (Bioline). Thermal profile initiated with a denaturation step at 95 °C for 5 min, followed by 40 cycles of amplification [95 °C for 30 s, annealing temperature (Ta °C) for 30 s, and 72 °C for 30 s] and a concluding elongation stage at 72 °C for 5 min. This gene amplification cycle was followed by a melting curve run, of 61 cycles with 0.5 °C increment from 65 to 95 °C. The primer information related to polyphenolic and carbohydrate biosynthetic genes (50–65 °C) are listed in Supplementary Table S1. Loquat's *actin* gene was used as a housekeeping reference gene (*ACT*).

To assess the transcript profile of *CWI2, CWI3, NI2, NI3,* and *SuSy,* primers designed for apple were used, since no specific primers for *E. japonica* are available. The selection of *Malus* was based on its close relation with *Eriobotrya* (both members of the Rosaceae family) and wide availability of published genome sequences. These were crossed-referenced after performing bioinformatics analysis on the NCBI database (http://www.ncbi.nlm.nih.gov/) that resulted in 100% primer similarity.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) according to Duncan's multiple way test with a significance level of 5% ($P \leq 0.05$) was used to compare mean values of total and individual sugars. The statistical package SPSS v.17.0 was employed.

Relative quantification and statistical analysis of gene expression levels was performed using REST-XL software with the employment of the pairwise fixed reallocation randomization test (Pfaffl et al., 2002).

3. Results and discussion

3.1. Incidence of purple spot

Fruit growth of all the three examined cultivars followed a typical sigmoid curve (Supplementary Fig. S1). Almost 60% of harvested fruit from the cultivar 'Obusa' presented PS symptoms, from the early on-tree developmental stages to culminate with the progress of fruit maturation (Table 1; Fig. 2). 'Karantoki' fruit with PS symptoms accounted instead for 31.3% of the harvested fruit; this percentage is significantly higher than other growing seasons and is attributed to adverse climatic

Table 1
Purple spot incidence (%) on each category (SI 1–5)[a] and severity index of the examined cultivars (cvs. 'Morphitiki', 'Karantoki', 'Obusa') at fully-ripe stage.

Purple spot incidence (%)		Cultivars		
		Morphitiki	Karantoki	Obusa
0–10%	SI-1	100%	68.7%	41.4%
10–30%	SI-2	0%	19.4%	11.2%
30–50%	SI-3	0%	9.6%	19.9%
50–70%	SI-4	0%	2.2%	16.9%
>70%	SI-5	0%	0%	10.6%
Total		100%	100%	100%
Severity Index		-	0.12	0.51

[a] SI stands for severity incidence of purple spot.

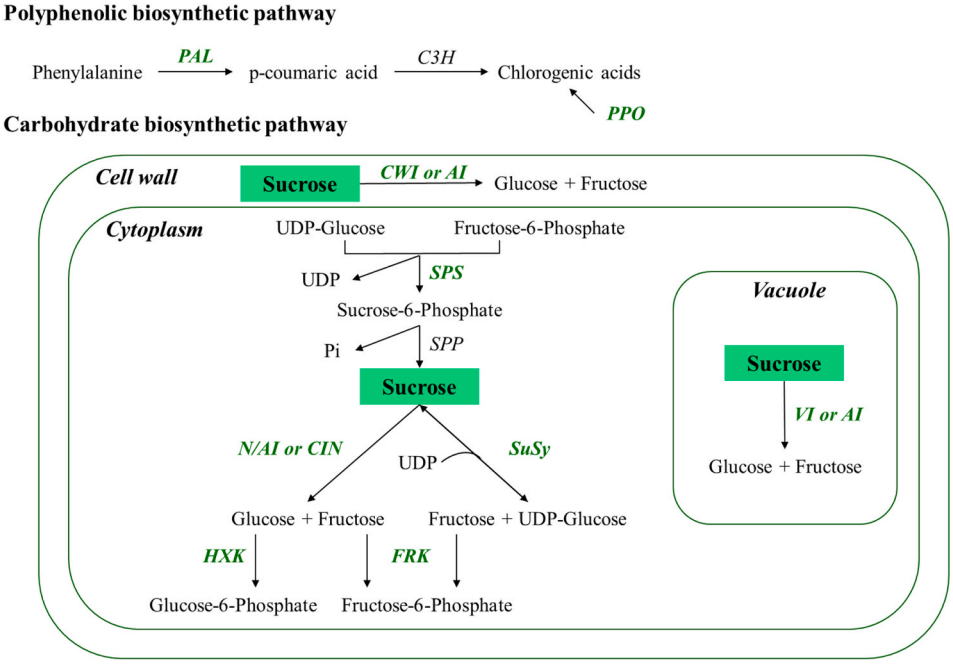

Fig. 1. Polyphenolic and carbohydrate biosynthetic pathways. Genes with expression levels studied are in italic green bold letters. The enzymes/genes are: *PAL, phenylalanine ammonia lyase; PPO, polyphenol oxidase; CWI, cell wall invertase; AI, acid invertase; SPS, sucrose phosphate synthase; SPP, sucrose phosphate phosphatase; N/AI, neutral/alkaline invertase; CIN, cytoplasmic invertase; SuSy, sucrose synthase; HXK, hexokinase; FRK, fructokinase; VI, vacuolar invertase.*
The metabolites are: phenylalanine; p-coumaric acid; chlorogenic acids; UDP-glucose; fructose-6-phosphate; sucrose-6-phosphate; sucrose; glucose; fructose; glucose-6-phosphate (Figure is modified from Nookaraju et al., 2010; Li et al., 2012; Busatto et al., 2014). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

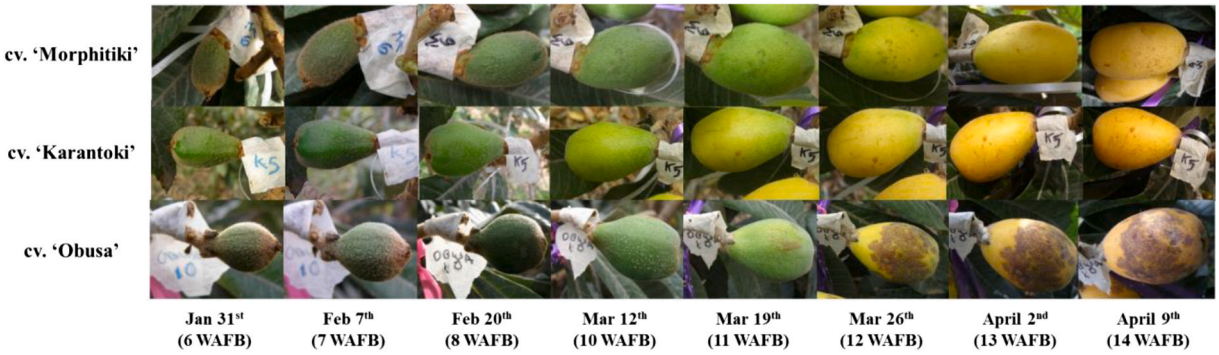

Fig. 2. Purple spot appearance during successive on-tree developmental stages of the examined loquat cultivars (cvs. 'Morphitiki', 'Karantoki' and 'Obusa'). 'WAFB' stands for weeks after full bloom. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conditions. 'Morphitiki' fruit instead did not show any visible PS symptoms (Table 1; Fig. 3). The Severity Index was 0.51 and 0.12 for 'Obusa' and 'Karantoki', respectively (Table 1). The high susceptibility to PS showed by the orange-fleshed loquat cultivar 'Obusa' has seriously limited its cultivation in Cyprus (Hadjipieri et al., 2017). However, for experimental purposes, this cultivar represents an excellent plant material for comparative purposes in order to decipher the PS physiological disorder. Notably, 'Karantoki' is the most widely cultivated loquat cultivar in Cyprus, due to its earliest harvest and bigger size; however, the intermediate susceptibility to PS disorder is particular evident in growing seasons with unfavorable climatic conditions (Goulas et al., 2014). Simultaneously, advances in genomic studies that are extensively applied in other species within the Rosaceae family are expected to offer new insights in loquat breeding programs through marker-assisted selection (Gisbert et al., 2009; Badenes et al., 2013). To this aim, the current study is aiming to step forward in the elucidation of sugar catabolism and selection of genes that can be considered as diagnostic markers of purple spot incidence and severity.

3.2. Total soluble sugars (TSS), sucrose, glucose, fructose contents

Soluble sugars such as sucrose (Suc), glucose (Glu) and fructose (Fru) are known to also act as key signal molecules regulating the expression of many genes involved in plant metabolic processes and defense responses, thus regulating plant growth and development (Li et al., 2012). Total soluble sugars ranged from 54.9 to 105.8 mg g^{-1} FW. Fructose (Fru) was the most abundant type sugar assessed with values ranging from 26.4 to 55.7 mg g^{-1} FW. Sucrose and glucose accounted instead for 11.2–22.8 mg g^{-1} FW and 12.4–20.0 mg g^{-1} FW, respectively, depending on the cultivar considered and the PS incidence.

'Karantoki' fruits with PS showed higher TSS, Suc, Glu and Fru contents compared with 'Obusa' (both asymptomatic and with PS symptoms) and 'Morphitiki' fruit. A slight increase in TSS, Glu and Fru contents for cv. 'Obusa' with PS was also observed in comparison with asymptomatic 'Obusa' fruit peel, although interestingly, the content of Suc was lower (Fig. 4). This decrease can be attributed to the fact that invertases break down Suc to Glu and Fru (Fig. 1). Carbohydrate content is a key factor determining fruit quality during maturation. Several studies showed that, in ripe loquat flesh tissue samples, Glu and Fru are the most dominant types of sugars (Song et al., 2016; Cai et al., 2019). In our study, we observed that peel with PS symptoms was characterized by a higher carbohydrate content with regards to portion of asymptomatic peel isolated from the same fruit. Gariglio et al. (2008) also reported that the concentration of sugars in the peel of loquat with PS was higher than in unaffected fruit. We hereby postulate a potential role of sucrose catabolism in the regulation of PS in peel of loquat fruit.

3.3. Gene expression profile

Thirteen candidate genes involved in key steps in the polyphenolic and carbohydrate biosynthesis pathways were transcriptionally assessed over the three examined cultivars. In particular, the relative expression

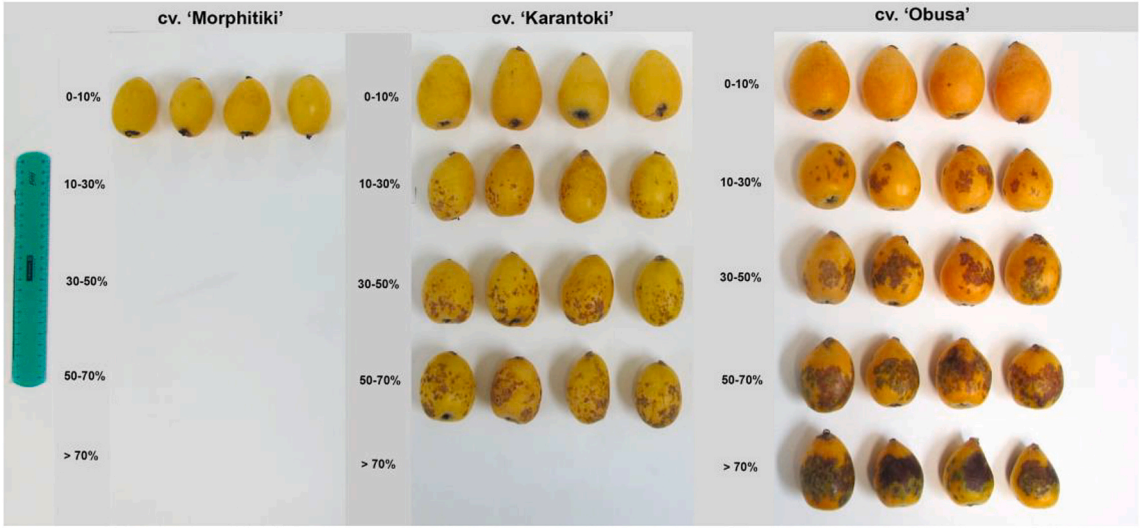

Fig. 3. Severity Incidence (SI) categorization for purple spot appearance on cvs. 'Morphitiki', 'Karantoki' and 'Obusa' loquat cultivars at fully-ripe stage. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

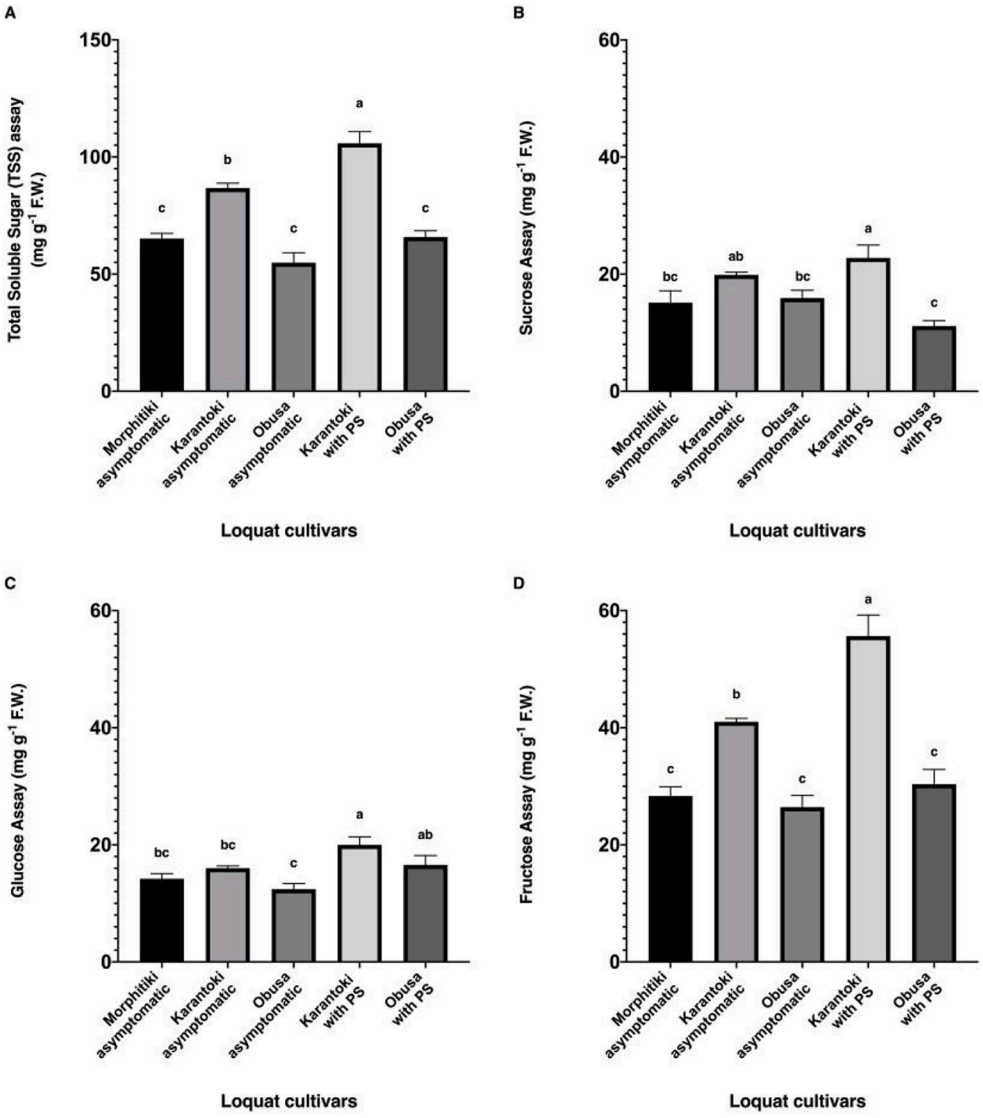

Fig. 4. Total soluble sugar (A), sucrose (B), glucose (C) and fructose (D) contents at fully ripe stage in loquat fruit peel of cvs. 'Karantoki' and 'Obusa', on both symptomatic (30–50%) and asymptomatic peel tissue of the same fruits at fully-ripe stage, as well as cv. 'Morphitiki' asymptomatic peel tissue at fully-ripe stage Values followed by the same letter are not significantly different according to Duncan's multiple range test at significance level 5% ($P \leq 0.05$). Data are the means of three biological replications ± SE.

levels of carbohydrate (*CWI2, CWI3, SPS1*, *SPS2, NI2, NI3, SuSy, HXK, FRK* and *VI*) and polyphenolic (*PAL1, PAL2* and *PPO1*) biosynthetic genes was assessed in peel tissues of both, symptomatic and asymptomatic loquat fruit, and depicted by means of heat map. Relative gene expression values were determined using cv. 'Morphitiki' asymptomatic peel tissue as a calibrator (Fig. 5, Supplementary Table S2).

With regard to the polyphenolic biosynthesis pathway, *PAL1* showed enhanced expression levels in both symptomatic (3.4-fold) and asymptomatic (4.0-fold) 'Obusa' fruit peel, while 'Karantoki' fruit showed reduced expression levels compared to 'Morphitiki' fruit that was used as reference. This trend suggests that *PAL1* may have an expression related to the different genetic background of the examined cultivars, without having any role in the PS development. Expression of *PAL2* seems to be instead more constitutive, not showing any particular modulation with regards to the development of PS. The last polyphenolic related gene tested was *PPO1*, a gene encoding for a polyphenol oxidase. Interestingly, its expression was lower in asymptomatic tissues of cvs. 'Karantoki' and 'Obusa', compared with tissues with PS. The latter can be partially associated with the role of *PPO* in controlling the superficial scald disorder in apple (Busatto et al., 2014, 2018). However, further and direct molecular approaches are needed in order to dissect whether common physiological regulation of the discoloration process occurring during the onset of these two fruit disorders exist. Based on our data, it remains unclear whether the etiology of purple spot disorder in loquat fruit can be assigned to an oxidative process triggered and coordinated by the action of the PPO enzyme, in a similar fashion to what has been observed in apple.

For the candidate genes involved in the carbohydrate pathway, the expression profile of ten genes was assessed. From the transcriptional profile depicted in the heat map (Fig. 5), a cultivar dependent pattern for a specific subset of genes has been observed. *SPS2, NI2 and HXK* are almost constitutive among the samples investigated herein. All these three genes showed a slight increment in expression levels for both asymptomatic or with PS symptoms cultivars ('Karantoki' and 'Obusa') in comparison with 'Morphitiki', yet not statistically significant.

SPS1 registered the highest expression levels *in* cv 'Karantoki' fruit with PS symptoms, aligned with a highest sucrose content (Figs. 4B and 5). *NI3* was more expressed in cv 'Karantoki', especially in fruit characterized by the onset of PS. Enhanced *NI* transcript levels that are associated with metabolism of Suc to Glu and Fru, may partially explain the increased Fru levels in 'Karantoki' fruit with PS. However, *NI3* does not appear to be directly associated with PS incidence since 'Obusa' fruit with PS did not register statistically significant differences compared with asymptomatic 'Morphitiki' fruits. Therefore, as for *NI2*, we may conclude that *NI3* follows a cultivar-dependent pattern without an evident correlation with sugar metabolism.

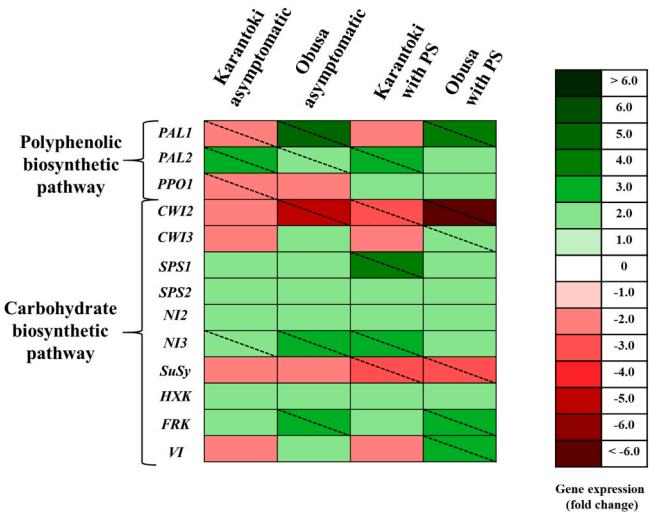

Fig. 5. Relative expression levels of polyphenolic biosynthetic genes (*PAL1, PAL2* and *PPO1*) and carbohydrate biosynthetic genes (*CWI2, CWI3, SPS1, SPS2, NI2, NI3, SuSy, HXK, FRK* and *VI*) in loquat fruit peel tissue of cvs. 'Karantoki' and 'Obusa', on both symptomatic (30–50%) and asymptomatic peel tissue at fully-ripe stage (n = 3). Relative mRNA expression levels were evaluated by real-time RT-qPCR using three biological repeats. A diagonal line in a box indicates a statistically significant value ($P \leq 0.05$). A scale of colour intensity is presented as a legend. Up-regulation is indicated in green; down-regulation is indicated in red. Relative gene expression values were determined using cv. 'Morphitiki' asymptomatic peel tissue as a calibrator. Actual relative expression levels are shown in Supplementary Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

On the other hand, *SuSy* resulted to be suppressed in both cvs. 'Karantoki' and 'Obusa' with PS. A down-regulation of *SlSuSy* and *SlLin5* (*CWI*) has been associated with the higher sucrose content in tomato fruit treated with xenobiotics (Christou et al., 2019), in line with their function as enzymes catalyzing the cleavage of sucrose into glucose and fructose [reversibly for SuSy, Stein and Granot (2019); irreversibly for invertase, Fotopoulos (2005)]. Overall, the fact that *SuSy* registered significantly lower expression levels in symptomatic tissue of both cultivars with PS symptoms suggests to be further examined as potential physiological marker of PS incidence.

The highly susceptible loquat cultivar 'Obusa' was distinguished by the enhanced expression pattern of three genes, namely *FRK, VI* and *CWI3*. Fructokinase (FRK) has an important role in the utilization of Fru. In the current study, *FRK* enhanced expression levels is not necessarily in accordance with the fructose content recorded, especially in 'Karantoki' peel with PS symptoms (Fig. 4). Furthermore, enhanced expression levels of FRK have been reguistered in asymptomatic tissue of 'Obusa'; therefore, its expression is cultivar specific rather than associated with purple spot. On the other hand, acidic invertase expression that involves *CWI3* and *VI* was substantially higher in 'Obusa' peel tissue with PS. *CWI3* can be considered as the dominant isoform gene for loquat mature fruit, in comparison with *CWI2*. The enhanced expression levels of *CWI3* and *VI* in the highly susceptible 'Obusa' peel tissue (Fig. 3), aligned with the catabolism of sucrose to glucose, are highly associated with the incidence of PS appearance.

4. Conclusions

Low temperature during the last on-tree developmental stages have been empirically connected with increased incidence and severity of PS disorder at the beginning of the harvesting season. Apart from climatic conditions, cultivar possesses a critical role in the incidence and severity of PS disorder. With the aim to shed some light on the incidence of PS disorder, thirteen genes involved in the polyphenolic and carbohydrate biosynthetic pathways in loquat fruit of three cultivars were examined. Acidic invertase levels involving *CWI3* and *VI* were substantially up-regulated in the symptomatic tissue of 'Obusa' fruits, which showed the highest overall sensitivity to the disorder, even from the colour breaking stage. The induction of *CWI3* and *VI*, aligned with catabolism of sucrose to glucose, provide potential molecular and metabolic markers for the cellular detection of PS. This is also the case for *sucrose synthase* (*SuSy*) that notably registered the lowest expression levels in both cultivars that demonstrated PS symptoms. Regarding the polyphenolic pathway, *PPO* may be associated with PS incidence but it needs to be further elucidated whether the etiology of the disorder can be assigned to an oxidative process triggered and coordinated by its action. Future experimental approaches should encompass global transcriptomic and metabolomic studies towards the further elucidation of sugar metabolism as well as the role of other physiological processes that might have a putative role in the incidence of purple spot.

CRediT authorship contribution statement

Margarita Hadjipieri: Formal analysis, Data curation, Investigation, Methodology, Writing - original draft. **Egli C. Georgiadou:** Formal analysis, Data curation, Investigation, Methodology, Writing - review & editing. **Fabrizio Costa:** Writing - review & editing. **Vasileios Fotopoulos:** Conceptualization, Supervision, Writing - review & editing. **George A. Manganaris:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.06.043.

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The impact of genotype and harvesting day on qualitative attributes, postharvest performance and bioactive content of loquat fruit

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ABSTRACT

Due to scalar on-tree ripening, harvest of loquat fruit is successive, spanning for several weeks, depending on the cultivar considered and the cultivation practices applied. Notably, early harvested fruit receive appreciably high prices on the market. The aim of the current study was to dissect the effect of harvesting day on mechanical properties, postharvest performance and phytochemical attributes (free and bound phenolic compounds and antioxidant capacity) of the predominant loquat cultivars grown in Cyprus (cvs. 'Karantoki' and 'Morphitiki'). Determination of the aforementioned attributes at harvest (H) and after additional maintenance at room temperature for 3 days (H + 3) for four successive harvesting dates (H1-4) were determined. Flesh firmness was slightly higher in early-harvested compared to late-harvested fruit, while slight or no differences after shelf life period for both cultivars were monitored. 'Karantoki' fruits manifested higher values of ripening index (SSC/TA) than 'Morphitiki'; such values were higher with the progress of harvest date due to a significant decrease of titratable acidity. 'Morphitiki' fruits were generally characterized by higher phenolic content, along with higher antioxidant capacity. No evident differences were registered between the harvest date and the shelf life period for both cultivars regarding free phenolic content. This study also highlights the significance of bound phenolics that contribute to the phenolic fraction of loquat fruit by 21.6–37.5%, depending on the cultivar and storage condition applied. Overall, the current study sheds light in the unexploited area of phytochemical properties of loquat fruits derived from successive harvesting dates.

1. Introduction

Loquat is a subtropical evergreen tree, originating from temperate China. Nonetheless, its global outspread started from Japan and since then it has undertaken diffusion in other markets (Tian et al., 2011). Its unique reversed annual cycle enables this species to satisfactorily adapt in the Mediterranean basin and other subtropical climates (González et al., 2010). The main countries of loquat production are China and Spain; the latter is the leading exporting country (Caballero and Fernández, 2004). In Cyprus, loquat cultivation is mainly favoured in the coastal regions. Despite its adaptability, a limited area is being cultivated, while *ca.* 30% of total area is covered by mesh and/or plastic to secure production volumes and advance fruit ripening. 'Karantoki' and 'Morphitiki' are considered as indigenous and/or traditional cultivars and are the most predominant, yet largely uncharacterised regarding their pomological properties (Goulas et al., 2014; Hadjipieri et al., 2019). Therefore, the evaluation of local germplasm and indigenous cultivars stands as an emerging need.

Loquat commercial production has risen in interest as it has stepped out from a small local cultivation to a niche product that receives appreciably high prices (Liu et al., 2016), particularly the early harvested fruit. This is mainly attributed to the lack of other fleshy fruits in the market during its harvesting period. Overall, loquat can potentially be identified as a promising fruit for the market, since traditional fruit crops in the Mediterranean climates are saturated (Chalak et al., 2014). However, its marketability is affected by purple spot appearance; a preharvest physiological disorder that leads to discoloration of the skin tissue without any effect in the inner fruit flesh. This disorder can affect up to 30% of the fruit's skin appearance, thus rendering them inappropriate for the market (Gariglio et al., 2002).

During fruit maturation, the physiology of loquat is evolving with the fruit size showing a rapid increase between the breaker (BBCH

Abbreviations: SSC, Soluble solids content; TA, Titratable acidity; H, Harvest day; H + 3, Three days shelf life; RI, Ripening Index; FF, Flesh Firmness; WL, Weight Loss; FW, Fresh Weight; GAE, Gallic acid equivalents; PAs, Phenolic Acids; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TE, Trolox equivalent; FP, Free phenolic content; BP, Bound phenolic content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power; PS, Purple Spot

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phenological stage 801 based on Martinez-Calvo et al. (1999) and half ripe stage (BBCH stage 805). The full colour is acquired at the ripe stage (BBCH stage 809) and based on the cultivar may differ from pale yellow to deep orange (Hadjipieri et al., 2017).

Loquat fruit remains rather unexploited regarding its phytochemical properties, since relatively few studies exist concerning its health-promoting benefits. Zhang et al. (2015) found that hydroxycinnamic acid and flavonols are the main constituents of loquat phenolic fraction. The fluctuation of the total phenolic content of 'Mogi' fruits throughout successive fruit developmental stages were mainly linked with changes occurring in the chlorogenic acid concentrations (Ding et al., 2001). Furthermore, variations on the total phenolic content and the antioxidant capacity among different genotypes exist (Ercisli et al., 2012) that can partially be attributed to their flesh coloration (Zhang et al., 2015). Other studies identified cultivars with higher phenolic and flavonoid contents which can be promoted as of high nutritional value and thus beneficial to human health (Ferreres et al., 2009; Xu and Chen, 2011; Xu et al., 2014). However, such studies have monitored the total phenolic content without taking into account the contribution of bound phenolic content, that only recently its contribution was acknowledged. Concomitantly, there has been no investigation on the harvesting period and how it is affecting the fruit phytochemical properties. Su et al. (2014) postulated that phenolic compounds exist both in free and bound forms in the cells of the plants and in contrast with the free phenolic compounds the bound ones, localized in the Cell wall matrix, cannot be extracted with water or aqueous/organic solvents mixtures.

The aim of the current study was to dissect changes on qualitative attributes of two indigenous loquat cultivars grown in Cyprus in correlation with the harvest period, through the evaluation of mechanical properties, qualitative attributes, postharvest performance and bioactive content, with special reference to both free and bound phenolic compounds.

2. Materials and methods

2.1. Fruit material and experimental design

Loquat fruits (cvs. 'Karantoki' and 'Morphitiki') were harvested from a commercial orchard under mesh, located in the Episkopi region, in the south coast of Limassol, Cyprus (34° 40′ 15′ N, 32° 54′ 7′ E). Three trees per cultivar with contiguous vigor, shape, age and maturity stage were harvested at four successive commercial harvesting dates for cv. 'Karantoki' and three for cv. 'Morphitiki' in 2016, based on fruit size and external color (March 4th, March 18th, April 1st and April 12th), designated as H1, H2, H3 and H4, respectively. Total yield per harvest as well as purple spot (PS) severity was registered. Subsequently, homogeneous lots of 60 representative sound fruits (subdivided into two 30-fruit sublots) per cultivar and harvest period were selected for further analysis. Sublots were analyzed upon harvest and after maintenance for 3 days at room temperature (shelf life). Each sub-lot was divided into three groups of 10 fruits; weight, color parameters (L*, a*, b*, Chroma and hue angle), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) were determined. Additionally, flesh tissue was flash frozen, homogenised to fine powder with liquid nitrogen and kept at −80 °C until needed for phytochemical analysis.

2.2. Qualitative attributes

Weight loss (WL) % was calculated as follows: (A–B) / A × 100, where A was the fruit weight at harvest and B was the fruit weight at H +3. Color was determined using the CR-400, Konica Minolta reflection colorimeter to indicate the coordinates L* (brightness or lightness; 0 = black, 100 = white), a* (−a* = greenness, +a* = redness) and b* (−b* = blueness, +b* = yellowness). Two measurements were made diametrically from equatorial sites of the fruit as technical replicates for both H and H + 3 for the same fruit. Hue angle [(H°) (0° =red-purple, 90° = yellow, 180° = bluish-green, 270° = blue)] and Chroma (degree of departure from grey to pure chromatic color) were also calculated as $\tan^{-1}$ (b*/a*) and $(a^{*2} + b^{*2})^{½}$ respectively. The ratio a*/b* was also calculated.

Flesh firmness (FF) was measured in two diametrical points on the equatorial region in 30 fruits from each cultivar, for both treatments with a texture analyzer (TA.XT plus, Stable Micro Systems, Surrey, U.K.), using a 3 mm diameter probe at a speed of 1 mm/s with a penetration depth of 5 mm and results were expressed in Newtons (N).

A professional juicer was used in order to extract the juice out of 10 fruits (three replicates for each treatment). For the quantification of the SSC, a PAL refractometer (ATAGO, PR-32α) was employed. TA was determined with the use of an automatic titrator (DL22 Mettler Toledo titrator, Mettler-Toledo, Inc., Columbus, Ohio, USA); briefly for each measurement, 5 mL of juice was used for titrating 0.1 N NaOH to a pH end point of 8.1. Results were expressed as g malic acid per 100 g fresh weight (FW). Ripening index (RI) was calculated as the SSC/TA ratio.

Total yield per harvest and cultivar considered was registered and the subsequent percentages of fruit yield per harvest were determined.

2.3. Extraction and determination of free and bound phenolics

Five grams of fine powdered flesh tissue was homogenized with 15 mL of 950 mL L^{-1} cold ethanol, vortexed and centrifuged at 4223 × *g* for 10 min. The pellet was then re-extracted with 10 mL of 800 mL L^{-1} cold ethanol. The supernatants were combined to make a final volume of 25 mL (Cao et al., 2009). Following the free phenolics extraction, 10 mL of 4 M NaOH were added in the pellet. The suspension was then sonicated for 90 min at 40 °C. Following alkaline hydrolysis, the solution was acidified to pH 2.0 with concentrated HCl. Then, the mixture was centrifuged at 4223 × *g* for 5 min to remove the cloudy precipitates. The liberated phenolic acids (PAs) were then extracted in the clear solution 3 times with 15 mL ethyl acetate and the upper phase was transferred in clean falcon for a total volume of 45 mL. The pooled ethyl acetate extracts were then evaporated to dryness at 45 °C. Subsequently, the dry residue was dissolved in 5 mL of ethanol and sonicated for 15 min (Irakli et al., 2012).

Aliquots of 100 μL of free and bound phenolic extracts were reacted with 500 μL of Folin-Ciocalteu and 4.8 mL of deionized water. The mixture was left to set for 3 min. Then, 1 mL of saturated sodium carbonate and 3.5 mL deionized water were added and left for 1 h in the dark at room temperature. The absorbance was measured at 725 nm (TECAN, Infinite 200® PRO) and results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} FW.

2.4. Determination of total antioxidant capacity

DPPH scavenging capacity was determined according to Goulas et al. (2014); 500 μL of the free phenolics sample and 200 μL of bound phenolics were mixed with 2 mL DPPH 0.135 mM. The mixtures were then incubated in the dark for 30 min and the absorbance was measured at 517 nm. A standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared and results were expressed in μmol 100^{-1} FW.

The antioxidant capacity was additionally determined by ferric acid reducing antioxidant power (FRAP) method as elsewhere described (Goulas et al., 2014). Briefly, a sample containing 3 mL FRAP solution and 250 μL of the free phenolics or 100 μL of bound phenolics was incubated in water-bath (37 °C) for 4 min. A standard curve of Trolox was also prepared. The absorbance was measured at 593 nm and results were expressed as μmol Trolox 100 g^{-1} FW.

2.5. Statistical analysis

Statistical analyses [Two-way ANOVA and Duncan Post-Hoc at a 5% significance level ($P \leq 0.05$)] for weight loss, soluble solids content,

Table 1
Price range at farmer, wholesale and retail level, total yield (kg) in three representative trees per cultivar and its proportion (%) per harvest of 'Karantoki' and 'Morphitiki' fruits.

	Price (€)**			Yield (kg, %)	
Harvest*	Farmer	Wholesale	Retailer	Karantoki	Morphitiki
H1	7.0	7.5	9.0	20.7 (9.0)	–
H2	5.0	6.0	7.0	58.4 (25.3)	45.9 (18.4)
H3	4.5	5.5	6.5	47.2 (30.7)	114.5 (46.0)
H4	3.0	4.0	5.0	81.0 (35.0)	88.7 (35.6)

*H1, H2, H3 and H4 represent the harvest dates (March 4th, March 18th, April 1st and April 12th) of loquat fruits.
**Prices at farmer, wholesale and retail level are similar for both cultivars.

titratable acidity, ripening index, flesh firmness as well as phenolics, DPPH and FRAP (free and bound form) were carried out using the software package SPSS v22.0 (SPSS, Inc., Chicago, IL, USA).

3. Results & discussion

3.1. Quality attributes

As the harvest period was progressing, the fruit size for both cultivars was increasing with cv. 'Karantoki' registering higher mean weight values. Interestingly, almost half production (46%) of cv. 'Morphitiki' was harvested in a single harvesting day (H3) that can be considered as a comparative advantage of the cultivar. Early harvested 'Karantoki' fruits, which accounts for the 9% of the total yield, received appreciably higher prices compared the other harvest dates. Total yield was similar for both cultivars with the higher yields being registered at the last two harvest dates (65.7% for 'Karantoki' and 81.6% for 'Morphitiki', Table 1).

'Morphitiki' presented lower weight loss (3.8–6.7%) compared to 'Karantoki' (5.0–6.9%) fruit subjected to 3 d shelf life. Notably, weight loss values were lower during the last harvesting time (H4) for both cultivars (data not shown). Similar results have been also postulated by Guerra et al. (2009) in plum fruit, where late harvested fruit were characterised by less weight loss than fruit from early harvest.

A 3.5–5.6% of 'Karantoki' fruits per harvest were classified as unmarketable due to the incidence of purple spot (PS) disorder, while 'Morphitiki' fruits were free of symptoms (data not shown). Over the harvest time period, PS appearance is reduced, in accordance with earlier studies (Gariglio et al., 2002, 2008) which pointed out that early-maturing orchards had higher incidence of PS at the beginning of the season. Gariglio et al. (2002) also indicated that the effect of low temperature, especially in early-ripening cultivars, can be the main cofactor responsible for the PS appearance on the fruit. In our study, the relatively low incidence of PS towards the last harvesting days can be attributed to the warm climate conditions.

Flesh firmness (FF) slightly decreased with the progress of on-tree ripening for both cultivars ranging from 1.01 to 1.22 N (H) and 0.91–1.18 N (H + 3) for 'Karantoki' and 1.08–1.19 N (H) and 1.00–1.24 N (H + 3) for 'Morphitiki', respectively. However, advanced harvest followed by shelf life (H4 + 3) led to flesh softening for both cultivars ca. 0.9N for 'Karantoki' and 1.0N for 'Morphitiki' fruits while this was not the case among the rest harvesting and shelf life periods (Table 2). Extended storage of loquat fruits is also not desirable as for certain loquat cultivars may lead to further firmness reduction (Amorós et al., 2008). Previous study indicated that cv. 'Morphitiki' was characterized by firmer fruit with appreciably acceptable appearance in comparison with 'Karantoki' during shelf life both after harvest and after removal from cold storage (Hadjipieri et al., 2019).

Soluble solids content (SSC) slightly increased with the progress of harvesting day for 'Karantoki' cultivar (from 11.4° Brix at H1 to 12.4° Brix at H4), while titratable acidity (TA) markedly decreased for both cultivars. 'Morphitiki' fruits had higher TA values compared with 'Karantoki' fruits for all harvests. The dramatic decrease of TA during fruit's shelf life was also indicated in previous studies (Cao et al., 2009; Goulas et al., 2014; Hadjipieri et al., 2019). The ripening index (RI) varied significantly both between cultivars and among the successive harvesting days ranging from 16.4–45.1 and 16.1–25.3 at harvest up to 31.3–70.1 and 24.8–40.8 during shelf life for 'Karantoki' and 'Morphitiki' fruits, respectively. Despite the fact that loquat fruit is considered as a non-climacteric type fruit (Tian et al., 2011), substantial changes on qualitative attributes occurs that are not necessarily linked with fruit deterioration.

Color parameter a* had higher values after the shelf life period with more evident differences in 'Morphitiki' fruits. The a*/b* ratio also registered higher values after the three days shelf life, manifesting deeper fruit color compared with the freshly harvested fruits. The L* colour parameters were higher at harvest for 'Morphitiki', as a more luminous cultivar compared to 'Karantoki'. Colour saturation, based on Chroma and hue angle parameters, showed slight variations although the hue angle was higher at harvest for both cultivars compared with the corresponding values during shelf life periods. 'Karantoki' had higher hue angle values (87.1 ± 0.3 for H3 and 87.8 ± 0.3 for H4) in comparison with 'Morphitiki' (82.9 ± 0.6 for H3 and 83.7 ± 0.5 for H4); this can be partially explained considering that 'Karantoki' peel is yellow and 'Morphitiki' is orange (Table 3). Furthermore, the higher a*/b* ratio values of 'Morphitiki' is an indicator of higher coloration.

All aforementioned quality parameters can be used as indicators for harvesting loquat fruit (Pinillos et al., 2011; Cañete et al., 2015). In the current study, early harvest coincided with higher TA values and lower RI, while non-significant differences in FF were monitored (Table 2). Pinillos et al. (2011) reported that a minimum SSC value of 10° Brix, along with a preferable colour as harvest indicators while Besada et al. (2013) mentioned a SSC value of 11° Brix to ensure optimum consumer satisfaction. In our study, SSC values for both cultivars were well above the threshold value of 11° Brix. The RI registered elevated values after shelf life, particularly at advanced harvestings for both cultivars (Table 2). Overall, the harvesting period is of great importance as early harvest may lead to disordered sweet/acid equilibrium with the outcome of harvesting over-acid fruits; a late harvest can also lead to absence of acidity which results to poor fruit quality (Besada et al., 2013).

3.2. Phytochemical composition

Phenolic content of 'Karantoki' ranged from 40.4 ± 1.1 to 48.7 ± 2.8 and from 11.8 ± 0.8 to 27.3 ± 0.5 GAE mg 100^{-1} FW for free and bound phenolics, respectively. The respective values for 'Morphitiki' cultivar were in the range 46.0 ± 2.0 to 53.9 ± 0.2 and 19.1 ± 4.5 to 31.5 ± 2.7 GAE mg 100^{-1} FW for free and bound phenolics compounds. Free phenolics (FP) contributed by 63.6–78.4 % of total phenolic content for 'Karantoki' and 62.5–71.8 % for 'Morphitiki' (Fig. 1). Early harvest for both cultivars was linked with higher phenolic contents, both in their free and bound form. Gruz et al. (2011) mentioned that the decrease of bound phenolics during the progress of fruit maturation is due to their alteration to forms that are no longer detectable by spectrophotometry. They have also indicated that the reduction in free and bound phenolics is related to the decrease of primary metabolism substrates that leads to a decline of the phenolic compound biosynthesis. Contrarily, Kevers et al. (2014) postulated that dark cherries and red raspberries presented higher phenolic and antioxidant capacity at their fully ripe stage in comparison with other dark fruits that had ambiguous or no differences at the harvesting period, indicating also that the antioxidant capacity varied according to harvest time, maturity stage and cultivation practises. Ding et al. (2001) reported that at the ripe stage of seven Japanese loquat cultivars, the total phenolic content was highly variable, ranging from 81.8 up to 173.8 mg 100 g^{-1} FW. Chlorogenic acid was the predominant compound,

Table 2
Soluble solids content (SSC), titratable acidity (TA), ripening index (RI), flesh firmness (FF) values for 'Karantoki' and 'Morphitiki' fruit, at successive harvests and after additional maintenance at room temperature for three days. Results are the means ± standard error (n = 3 for SSC, TA and RI, n = 30 for FF).

Treatments	SSC (%)	TA (Malic acid, %)	Ripening Index	Firmness (Newtons)
cv. 'Karantoki'				
H1*	11.4 ± 0.1e**	0.69 ± 0.04a	16.4 ± 1.1fg	1.22 ± 0.05ab
H1 + 3d	11.9 ± 0.6de	0.39 ± 0.03c	31.3 ± 4.0de	1.18 ± 0.06abc
H2	12.0 ± 0.1cde	0.32 ± 0.01 cd	37.3 ± 0.9 cd	1.15 ± 0.05abc
H2 + 3d	12.5 ± 0.4abcd	0.23 ± 0.02de	54.3 ± 5. 3b	1.17 ± 0.04abc
H3	12.0 ± 0.2cde	0.30 ± 0.00 cd	39.6 ± 0.2 cd	1.09 ± 0.03 cd
H3 + 3d	12.9 ± 0.4abc	0.18 ± 0.00e	70.1 ± 1.3a	1.12 ± 0.04bcd
H4	12.4 ± 0.5bcd	0.27 ± 0.02de	45.1 ± 2.6c	1.01 ± 0.05de
H4 + 3d	13.3 ± 0.2ab	0.24 ± 0.03de	54.8 ± 6.9b	0.91 ± 0.02e
cv. 'Morphitiki'				
H2	12.0 ± 0.1cde	0.75 ± 0.04a	16.1 ± 0.8 g	1.19 ± 0.04abc
H2 + 3d	13.3 ± 0.3a	0.54 ± 0.04b	24.8 ± 1.7egf	1.24 ± 0.05a
H3	12.3 ± 0.2cde	0.52 ± 0.05b	23.4 ± 2.5efg	1.12 ± 0.03bcd
H3 + 3d	12.7 ± 0.2abcd	0.31 ± 0.02 cd	40.8 ± 1.9 cd	1.13 ± 0.03abc
H4	12.3 ± 0.2cde	0.49 ± 0.06b	25.3 ± 2.8ef	1.08 ± 0.04 cd
H4 + 3d	12.8 ± 0.3abcd	0.34 ± 0.01 cd	38.0 ± 2.0 cd	1.00 ± 0.03de

*Harvest dates designated as H1, H2, H3 and H4 are indicated in Table 1.
**Different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H + 3).

accounting for up to half of the total phenolics.

'Morphitiki' registered higher total phenolic content, mainly due to the higher bound phenolics content (Fig. 1). In an earlier study of our group, 'Morphitiki' also registered higher free phenolic content than 'Karantoki' (Goulas et al., 2014). Acosta-Estrada et al. (2014), indicated that the percentage of bound phenolics over the total phenolic content varies significantly, *i.e.* 6.5% in apple, 33.1% in banana, 24.3% in orange and 20.7% in medlar fruits. Comparing the bound phenolic contend of 11 common fleshy fruits, Sun et al. (2002) reported on average a 24% of the total content derive from the bound form. Based on their contribution in total phenolic fraction, the determination of bound phenolics needs to be taken into account.

In the current study, no evident differences between the harvest date and the shelf life period for both cultivars regarding the free phenolic content were monitored. The bound phenolics presented the highest values at H1 (25.8 ± 1.6 GAE mg 100^{-1} FW) and H1 + 3 (27.3 ± 0.5 GAE mg 100^{-1} FW) for 'Karantoki' cultivar. Similarly, 'Morphitiki' registered higher FP values at H2 and H3 (51.7 ± 0.4–53.9 ± 0.2) as well as BP values at the same harvesting days (24 ± 0.8–31.5 ± 2.7); higher values for both free and bound phenolics were registered at H2 + 3, 53.9 and 31.5 GAE mg 100^{-1} FW respectively (Fig. 1). Overall, bound phenolics registered a decrease at advanced harvested 'Karantoki' fruits whilst bound phenolics for 'Morphitiki' fruits had negligible changes during the harvesting period. The non-significant changes of bound phenolics in 'Morphitiki' fruits can potentially be correlated with ripening behavior as the SSC also was constant among harvests (Table 2). In general, the bound phenolics are covalently bound to sugar moieties or cell wall structural components that are released with the progress of ripening. It should be also taken into account that the Folin–Ciocalteu assay does not detect all phenolic groups found in the extracts and it cannot monitor the release of bound phenolics as free (Stalikas, 2007).

3.3. Antioxidant capacity

Antioxidant capacity was determined with the employment of DPPH and FRAP assays and results for both free and bound forms are presented. The DPPH and FRAP values for the free forms showed higher content in comparison with the bound forms due to their abundance in the free fraction. More specific the free DPPH values (μmol Trolox

Table 3
Color parameters (L*, a*, b*, a*/b*, chroma and hue angle) of 'Karantoki' and 'Morphitiki' fruits at successive harvests and after additional maintenance at room temperature for three days. Results are the means ± standard error (n = 30).

	Color parameters					
Treatments	L*	a*	b*	a*/ b*	Chroma	Hue angle
cv. 'Karantoki'						
H1*	60.1 ± 1.0 f**	2.2 ± 0.4g	46.0 ± 1.5bc	0.05 ± 0.01h	46.1 ± 1.5bc	86.7 ± 0.4ab
H1 + 3d	59.8 ± 0.6f	3.6 ± 0.4f	44.5 ± 0.5cd	0.08 ± 0.01fg	44.7 ± 0.5cd	85.4 ± 0.5c
H2	59.2 ± 0.8f	3.4 ± 0.4f	45.6 ± 0.5cd	0.08 ± 0.01g	44.8 ± 0.5cd	85.7 ± 0.5bc
H2 + 3d	63.0 ± 0.4cd	4.9 ± 0.3de	47.3 ± 0.4ab	0.10 ± 0.00ef	47.5 ± 0.4ab	84.1 ± 0.3de
H3	60.2 ± 8f	2.2 ± 0.2g	43.7 ± 0.5d	0.05 ± 0.01h	44.7 ± 0.5d	87.1 ± 0.3a
H3 + 3d	64.5 ± 0.4bc	4.1 ± 0.2ef	47.4 ± 0.4ab	0.09 ± 0.01fg	47.6 ± 0.4ab	85.1 ± 0.3cd
H4	63.5 ± 0.7bcd	1.4 ± 0.3g	43.6 ± 0.5d	0.03 ± 0.01h	43.6 ± 0.5d	87.8 ± 0.3a
H4 + 3d	60.3 ± 0.6f	3.3 ± 0.3f	42.8 ± 0.5d	0.08 ± 0.01g	42.9 ± 0.5d	85.5 ± 0.3bc
cv. 'Morphitiki'						
H2	65.0 ± 0.3ab	6.7 ± 0.3a	48.2 ± 0.4a	0.14 ± 0.01cd	48.8 ± 0.4a	81.9 ± 0.4fg
H2 + 3d	61.0 ± 0.5ef	8.3 ± 0.3a	46.8 ± 0.5ab	0.18 ± 0.01a	47.5 ± 0.5ab	79.9 ± 0.4i
H3	66.7 ± 0.4a	5.8 ± 0.5bc	46.8 ± 0.4ab	0.12 ± 0.01de	47.6 ± 0.3ab	82.9 ± 0.6ef
H3 + 3d	65.3 ± 0.3ab	8.1 ± 0.4a	46.9 ± 0.4ab	0.17 ± 0.01ab	47.7 ± 0.4ab	80.2 ± 0.5hi
H4	62.4 ± 0.8de	4.7 ± 0.4e	43.0 ± 0.5d	0.11 ± 0.01e	43.4 ± 0.5d	83.7 ± 0.5e
H4 + 3d	54.7 ± 1.0g	6.6 ± 0.4ab	39.7 ± 0.6e	0.15 ± 0.01bc	40.2 ± 0.6e	81.4 ± 0.5gh

*Harvest dates designated as H1, H2, H3 and H4 are indicated in Table 1.
**Different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H + 3).

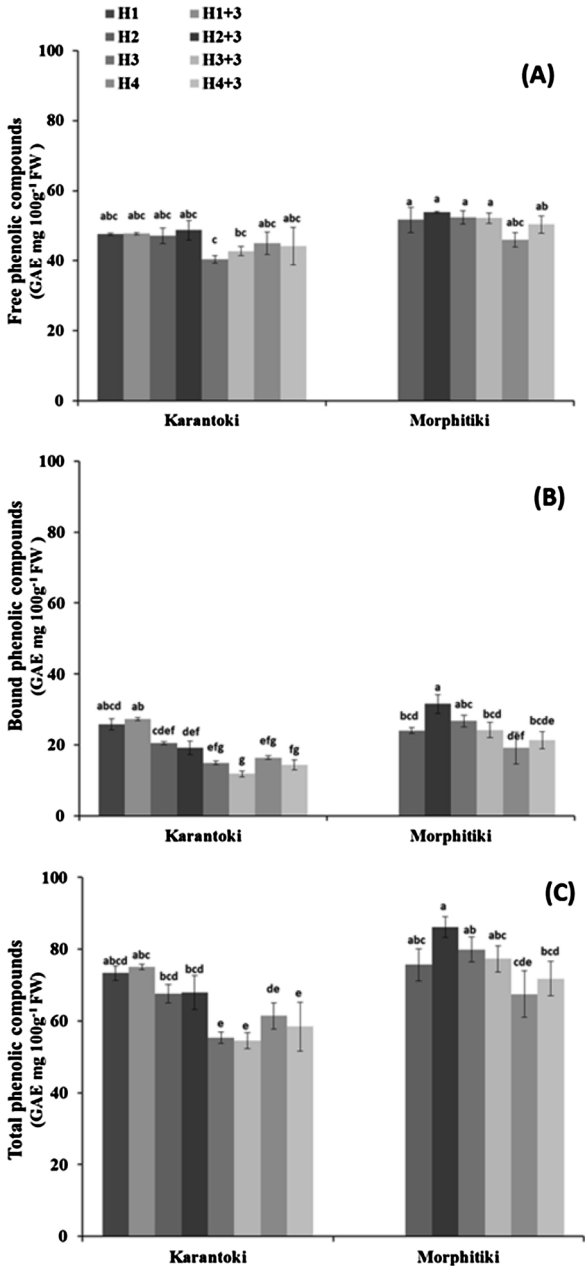

Fig. 1. Free (A), Bound (B) and total phenolic content (C) of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days. Harvest dates are indicated in Table 1. Results are the mean ± standard error (n = 3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).

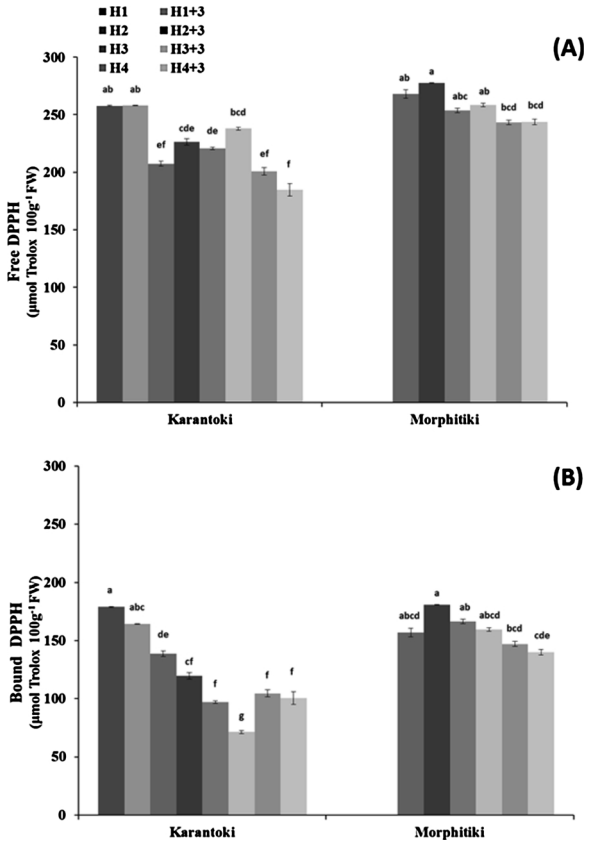

Fig. 2. DPPH free (A) and bound (B) antioxidant capacity of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days. Harvest dates are indicated in Table 1. Results are the mean ± standard error (n = 3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H+3).

100^{-1} FW) ranged from 184.6 ± 10.7 to 257.9 ± 6.9 and from 243.2 ± 3.4 to 277.7 ± 8.9, whereas the bound DPPH values ranged from 71.3 ± 4.9 to 179 ± 3.6 and from 140.2 ± 10.9 to 180.7 ± 6.6 for 'Karantoki' and 'Morphitiki', respectively (Fig. 2). Similarly, free FRAP values (µmol Trolox $100\,g^{-1}$ FW) ranged from 178.2 ± 17.1 to 226.1 ± 4.2 for cv. 'Karantoki' and 123.7 ± 2.3 to 148.7 ± 6.5 for cv. 'Morphitiki'. The bound FRAP values ranged from 52.8 ± 13.1 to 137.3 ± 2.7 and from 64.5 ± 6.7 to 144.1 ± 12.1 for cv. 'Karantoki' and cv. 'Morphitiki' respectively (Fig. 3). Goulas et al. (2014) postulated also higher DPPH and FRAP antioxidant capacity for the 'Morphitiki' cultivar in comparison with 'Karantoki', without however taking into account the bound form.

DPPH (free and bound) values registered the highest values during the early harvest plus shelf life for both cultivars. Bound FRAP had higher values for the H1 and H2 for the 'Karantoki' cultivar and their respective shelf life periods. Similarly, 'Morphitiki' had higher bound FRAP content at H2+3. For both cultivars, these values went descending after advanced harvesting days and the subsequent shelf life periods. (Figs. 2 and 3).

High positive correlation between the phenolic content and the antioxidant capacity is manifested in accordance with previous studies both for loquat fruits (Xu and Chen, 2011; Goulas et al., 2014; Zhang et al., 2015) and other fruit tissues (Gil et al., 2002; Sun et al., 2002; Kevers et al., 2014). The correlation coefficients (r) between phenolic content and antioxidant capacity were additionally calculated to dissect the main loquat components that are responsible for antioxidant capacity. A strong correlation was found between bound phenolics and DPPH as well as between bound phenolics and FRAP capacity (0.954 and 0.827), highlighting that significant amounts of antioxidant phenolics were revealed after hydrolysis. On the contrary, the correlation coefficients between total phenolics and antioxidant capacity in free fraction was lower (0.682 and −0.495). Such results can be linked with the potential presence of other groups of antioxidant compounds, such as carotenoids and ascorbic acid.

4. Conclusions

There are variations between the qualitative characteristics of the loquat fruit in relation to the harvest date and the cultivar considered. Early harvested 'Karantoki' fruits were characterized by less appreciable qualitative properties, evident by low a*/b* ratio, decreased SSC

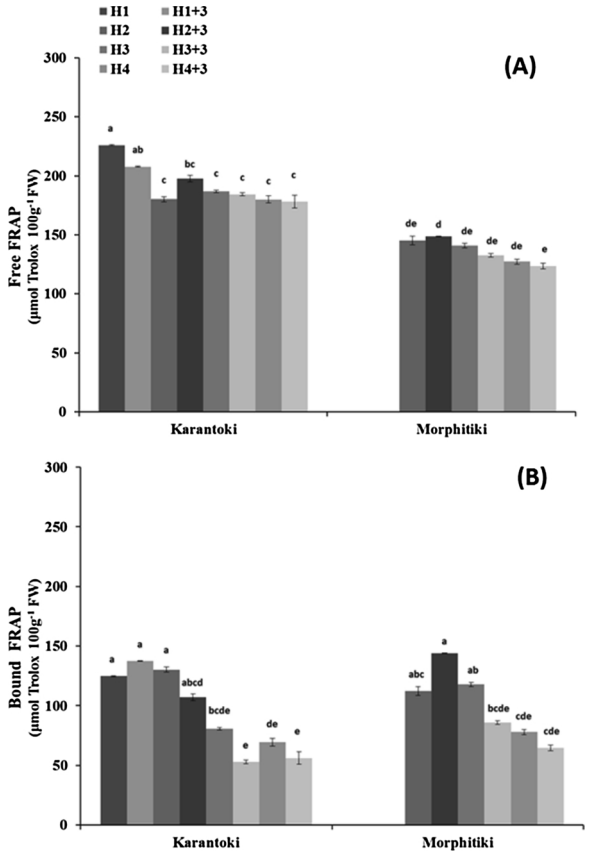

Fig. 3. FRAP free (A) and bound (B) antioxidant capacity of 'Karantoki' and 'Morphitiki' fruits at harvest (H1-H4) and after additional maintenance at room temperature for 3 days. Harvest dates are indicated in Table 1. Results are the mean ± standard error (n = 3) and different letters for each parameter shows the statistical difference between the two cultivars and the treatments applied (H and H + 3).

and elevated TA values. Early harvests and the corresponding shelf life periods for both cultivars were linked with higher phytochemical content compared to late harvests, evident by higher antioxidant capacity and bound phenolic content. A highly positive correlation between the bound phenolic content and the antioxidant capacity of loquat fruit was monitored. 'Morphitiki' was characterized by higher values for all harvest dates, highlighting the genotype significance.

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The effect of modified atmosphere packaging on postharvest performance of two loquat cultivars

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Abstract

Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is the only fruit tree crop in the Mediterranean zone with ripening period during early spring, particularly when specific preharvest practices are applied such as cultivation under plastic cover. Loquat fruit is characterised by its refreshing taste and it is highly appreciated by consumers. However, loquat fruit is characterised by relatively short storage potential partially due to senescence and/or chilling related disorders. The aim of the current study was the evaluation of Xtend® packaging as a means of modified atmosphere packaging (MAP) on postharvest performance of fruit from the main loquat cultivars grown in Cyprus, namely 'Karantoki' and 'Morphitiki'. Fruit were subjected to cold storage in conventional refrigerator (4°C) for 3 weeks and subsequently allowed at room temperature for 0, 2, 4 and 7 days, respectively. For each treatment, 30 fruits were used to determine weight loss, peel colour differentiations (CIEL*, a*, b*), flesh firmness (Texture analyser, Stable Micro Systems), soluble solids content (SSC) and titratable acidity (TA). 'Morphitiki' presented better phenotypic appearance compared to 'Karantoki' after extended storage, mainly due to reduced weight loss. Application of MAP significantly reduced weight loss after removal from cold storage. However, extended maintenance at room temperature (4 and 7 days) deteriorated the performance of both cultivars. As a technological perspective of the current study, data suggest that 'Morphitiki' fruit subjected to MAP can be refrigerated up to 3 weeks, provided they are consumed within two days after removal from storage.

Keywords: *Eriobotrya japonica,* cold storage, weight loss, soluble solids content, titratable acidity, chilling

INTRODUCTION

Loquat is a highly perishable commodity with relatively short shelf life period (Tian et al., 2007), while fruit can be cold stored at high relative humidity for up to 3 weeks (Kader, 2002). Due to its perishability, alternative methods to conventional cold storage (CS) with the aim to expand its market life were dissected such as: modified atmosphere packaging (MAP) (Ding et al., 2006; Pareek et al., 2014), controlled atmosphere (Ding et al., 2006), paper bags packaging and 1-methylcyclopropene (Zheng et al., 2010; Cao et al., 2011). The minimum safe cold storage temperature ranges from 0 to 10°C, highly dependent on the sensitivity of cultivar on chilling injury (CI) symptoms. Loquat fruits conditioned at 1°C for 30 days in polyethylene bags with small holes kept their original quality and chemical characteristics intact (Ding et al., 1998). Cold storage at 5°C in polyethylene bags of 20 µm width and with internal gas content 4% O_2 and 5% CO_2 showed better performance, while organic acids and carotenoid contents registered higher values compared to control (Ding et al., 2002).

Loquat cultivation in Cyprus is mainly based in two traditional cultivars, namely 'Morphitiki' and 'Karantoki' (Figure 1), for which relatively few data exist about their storage potential and postharvest performance. 'Morphitiki' has rounded to egg fruit shape, juicy white to yellow flesh with refreshing taste (Goulas et al., 2014). 'Karantoki' bears larger pear-shaped fruit and shows earlier fruit maturation (ca. 15 days earlier than 'Morphitiki'). MAP treatment has been successfully applied in an array of commodities; in the current study its effect in loquat fruit was dissected.

Figure 1. Mature 'Karantoki' (a) and 'Morphitiki' (b) fruits with distinct anatomical properties.

MATERIALS AND METHODS

Fruit material ('Morphitiki' and 'Karantoki') were harvested from a covered commercial orchard (Episkopi, Limassol, Cyprus), based on size uniformity and external colour. Fruit were initially used for the determination of physical dimensions and colour and subsequently for analytical studies as described below. For each cultivar and storage treatment, lots of 30 fruits (three 10-fruit sub-lots) were used. Fruit (control and MAP-treated) were placed into a conventional refrigerator at 4°C (CS) for 3 weeks and subsequently evaluated after 0, 2, 4 and 7 days shelf life, respectively. The specific packaging is used worldwide for storing an array of commodities, such as pomegranates, cherries, bananas, fresh vegetables, citrus and stone fruits (DECCO-UPL 2005). Weight loss (WL), colour parameters, flesh firmness, soluble solids content (SSC) and titratable acidity (TA) were determined.

Weight loss (WL) % was monitored as follows: (A-B)/A×100, where A was the fruit weight at harvest and B was the fruit weight after the shelf life period. The colour parameters CIE L* (brightness or lightness; 0 = black, 100 = white), a* (-a* = greenness, +a* = redness) and b* (-b* = blueness, +b* = yellowness) were measured in the peel tissue at both sides of each fruit, using a Minolta chromatometer (CR-400, Konica Minolta). Flesh firmness (FF) was determined on opposite sides of the equator of each fruit with a penetrometer (TA.XT plus, Stable Micro Systems, Surrey, UK) fitted with a 3-mm plunger at 1 mm s^{-1} speed and 5 mm depth; the two readings were averaged for each fruit and results expressed in g. Soluble solids content (SSC) of the juice was measured with a digital refractometer (DR103L, Sun Instruments Corp., USA) and data were expressed as %. Titratable acidity (TA) was measured using an automatic titrator (DL22 Mettler Toledo titrator, Mettler-Toledo, Inc., Columbus, Ohio, USA) and determined by titrating 5 mL of juice with 0.1 N NaOH to a pH end point of 8.1. Results were expressed as g malic acid 100 g^{-1} FW. Ripening index (RI) was calculated as the SSC/TA ratio.

Data were analysed based on the ANOVA method according to 2×1×8-9 factorial design (2 cultivars × 1 method × 8-9 treatments) with 30 replications for treatments combinations. Means were compared with the Duncan test at $P \leq 0.05$. All statistical analyses were carried out using the software package SPSS v22.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Modified atmosphere packaging led to reduced weight loss (Table 1), in accordance with similar studies in other loquat cultivars (Amorós et al., 2008). However, extended shelf life (4 or 7 days) after removal from cold storage led to substantially high weight loss in all cases. 'Karantoki' fruit showed higher weight losses after removal from cold storage, and intense shriveling after extended shelf life period (Figure 2).

Table 1. Weight loss (%), ripening index (SSC/TA), flesh firmness (g) of 'Karantoki' and 'Morphitiki' fruits at harvest and after refrigerated storage (cold storage, CS) with or without modified atmosphere packaging (MAP) and additional maintenance at room temperature (shelf life, SL) for 0, 2, 4 and 7 days, respectively. Results are the means ± standard error. The statistical analysis for each parameter shows the statistical difference between the two cultivars and the storage treatments.

Treatment	Weight loss (%)		Ripening index (SSC/TA)		Flesh firmness (g)	
	Karantoki	Morphitiki	Karantoki	Morphitiki	Karantoki	Morphitiki
Harvest			37.3±1.2g	19.3±2.5i	105.4±4.5f	130.1±7.2 cde
3 w CS + 0 d SL	3.5±0.2i	3.6±0.1i	67.6±5.0ef	35.7±8.8gh	125.3±4.7de	142.0±3.4 abcd
3 w CS + 2 d SL	7.5±0.4f	6.4±0.2g	77.9±1.5bcde	40.7±0.7g	142.0±9.6abcd	135.5±4.3 bcde
3 w CS + 4 d SL	8.7±0.3d	9.6±0.2e	83.6±2.0bcd	68.9±4.6ef	126.4±4.4 cde	133.5±4.9 bcde
3 w CS + 7 d SL	14.2±0.6a	10.5±0.3c	99.9±2.4a	76.6±2.1cde	119.0±6.8 ef	130.4±4.7 cde
3 w MAP + 0 d SL	0.7±0.04j	0.7±0.04j	63.1±3.07f	25.3±2.4fhi	145.3±5.6 abc	150.7±4.9 ab
3 w MAP + 2 d SL	5.0±0.2h	3.4±0.1i	77.8±4.6bcde	35.7±3.8gh	135.1±6.6 bcde	154.8±5.1a
3 w MAP + 4 d SL	9.3±0.3e	7.2±0.4f	88.3±2.1b	42.2±4.5g	139.2±5.5 abcd	150.6±5.4ab
3 w MAP + 7 d SL	15.0±0.5b	1.8±0.5d	86.2±3.82bc	73.9±0.6def	128.7±5.2 cde	141.7±3.1 abcd

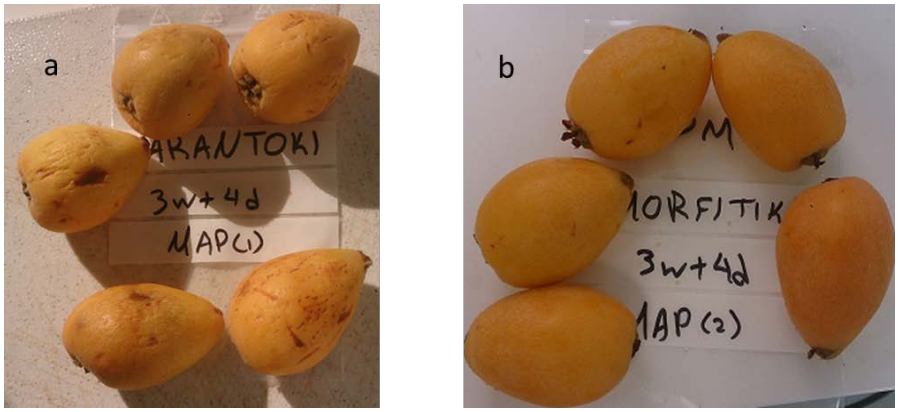

Figure 2. Phenotypic appearance of 'Karantoki' (a) and 'Morphitiki' fruits (b) after 3-week refrigerated storage under modified atmosphere packaging and additional maintenance at room temperature (shelf life, SL) for 4 days.

Flesh firmness registered higher values after removal from cold storage in both cultivars compared to harvest, both in control and MAP-treated fruit; 'Morphitiki' fruit presented higher firmness values than 'Karantoki' (Table 1). Notably, an increase in tissue firmness of specific loquat cultivars during postharvest was reported, being attributed to lignin biosynthesis (Cai et al., 2006); while in other loquat cultivars, extended storage (6 weeks) led to firmness reduction (Amorós et al., 2008).

Ripening index increased with the progress of shelf life period (Table 1). 'Karantoki' fruit possessed higher values, mainly due to lower titratable acidity, particularly after extended shelf life, as elsewhere described (Goulas et al., 2014). Both cultivars presented higher RI after CS with or without MAP treatment after shelf life of 7 days. Modifications in SSC were less intense compared to TA during the shelf life period for both cultivars (data not shown).

Regarding colour parameters, L* values went descending compared to harvest in both cultivars (Table 2). According to Amorós et al. (2008), L* values reduced in loquat fruit after CS treatments and subsequent maintenance at room temperature with only a slight decrease when the fruit was treated with MAP and then maintained at room temperature. The a* parameter showed an increase during SL after removal from CS, registering the highest values in 'Morphitiki' fruits after postharvest maintenance for 4 and 7 days, both in control and MAP-treated fruits. This is a typical phenomenon, also monitored in other loquat cultivars where

the a* parameter was increased during SL after CS treatment (Amorós et al., 2008). The b* values went descending compared to harvest in both cultivars for both treatments. According to Amorós et al. (2008), the b* parameter showed a decrease during CS as well as CS and SL, while MAP treatment did not affect it. Notably, the ratio a*/b* in our study indicated that the 'Morphitiki' cultivar had a more desirable colour which, over time, got deeper but was not affected by the MAP treatment as it showed no statistical difference in all corresponding treatments (Table 2).

Table 2. Colour parameters for 'Karantoki' and 'Morphitiki' fruits at harvest and after refrigerated storage (cold storage, CS), with or without modified atmosphere packaging (MAP) and additional maintenance at room temperature (shelf life, SL) for 0, 2, 4 and 7 days, respectively. Results are the means ± standard error. The statistical analysis for each parameter (L*, a*, b* and a*/b* ratio) shows the statistical difference between the two cultivars and the treatments.

Treatment	L*		a*		b*		a*/ b*	
	Karantoki	Morphitiki	Karantoki	Morphitiki	Karantoki	Morphitiki	Karantoki	Morphitiki
Harvest	64.6± 0.4b	67.7± 0.3a	2.6± 0.4jk	5.1± 0.4efg	46.0± 0.6b	48.5± 0.6a	0.06ij	0.11 gh
3 w CS + 0 d SL	49.8± 1.1gh	48.4± 1.2h	3.2± 0.2ij	5.5± 0.3def	38.7± 0.8hi	36.9± 0.7i	0.09hi	0.15cd
3 w CS + 2 d SL	53.8± 0.9def	53.9± 0.9def	4.1± 0.2ghi	6.8± 0.3b	40.5± 0.7fgh	41.6± 0.7efg	0.10 gh	0.17bc
3 w CS + 4 d SL	54.7± 0.8cdef	55.9± 0.6cde	4.8± 0.8efgh	8.1± 0.3a	42.4± 0.6def	43.8± 0.6cde	0.11fg	0.19b
3 w CS + 7 d SL	54.9± 1cde	53.1± 1.2ef	6.2± 0.3bcd	8.9± 0.3a	44.5± 0.8bcd	42.0± 1.3ef	0.14def	0.22a
3 w MAP + 0 d SL	56.3± 0.8cd	53.7± 1.0def	2.8± 0.2jk	5.6± 0.4cde	42.1± 0.6ef	40.3± 0.7fgh	0.07ij	0.14def
3 w MAP + 2 d SL	51.8± 0.9fg	49.0± 1.3h	3.9± 0.2hi	6.6± 0.4bc	40.3± 0.6fgh	38.9± 0.8hi	0.10 gh	0.17bc
3 w MAP + 4 d SL	49.2± 1.0gh	57.4± 0.8c	4.6± 0.2efgh	8.1± 0.4a	39.3± 0.7gh	45.3± 0.8b	0.12efg	0.18b
3 w MAP + 7 d SL	56.3± 0.9cd	58.2± 1.3cde	6.4± 0.4bcd	8.9± 0.4a	44.6± 1.0bcd	42.6± 1.4def	0.14cde	0.22a

CONCLUSIONS

Results indicated the beneficial effect of MAP in maintaining postharvest appearance of loquat fruit, even under extended conventional refrigerated storage, provided that they will be consumed in a short period after removal from storage. Furthermore, 'Morphitiki' fruits presented appreciable better appearance compared to 'Karantoki' fruit; the latter were characterized by high levels of weight loss that led to both quantitative and qualitative losses, particularly after extended shelf life periods (Figure 2). Therefore, the significance of genotype is also highlighted in the current study.

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