

# **Anatomical Studies on the Accumulation of Biopolymers in Various Fruit Tree Roots**

**TULADHAR ASTHA**

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## General Introduction

Water availability is an important factor for planning a long term commercial orchard because it can directly affect root growth and maintenance of tree vigor. Understanding tree physiology can help us maximize tree vigor so that stable production becomes possible over the years. Fruit trees, unlike herbaceous crops, are hardly transplanted. As they mature, fruit trees develop a wide-spreading root system that fixes them to one place where they must constantly adapt to local soil environment including stressful conditions like temporary or long term water-logging or drought. An important feature of fruit trees is that their roots become woody as they age. Many questions regarding how woody roots adapt to unfavorable growth conditions remain unanswered today. Although studying roots below ground is challenging due to limited visibility, a complete understanding of tree physiology is not possible without studying the adaptive responses of root cells.

The cell wall which is unique to plant cells is considered as the first compartment to be affected by stress signals (Komatsu et al., 2010, Salvador et al., 2013). It is known that biopolymers such as lignin and suberin accumulate in the plant cell wall to play an important protective role (Moerschbacher et al., 1900, Nelson and Wilhelm, 1957, Williamson, 1984). So far, lignin and suberin accumulation has been observed in plant body parts such as the bark (Hawkins and Boudet, 1996), the fruit (Gong et al., 2013), the leaf (Poovaiah, 1974) and also in roots (Steudle and Peterson, 1998). However, details on biopolymer accumulation in woody roots and changes occurring due to stress remain unexplored.

Water intake by roots is crucial for plant survival. However, drought and water-logged conditions may hamper root function and crop production at large. Studies reveal that anatomical changes are observed when roots encounter drought conditions (Shao et al., 2008) and water-logged conditions (Armstrong et al., 1994; Schussler and Longstreth,

2000). Tardieu et al. (1991)'s report that drought influences the ABA concentration in the xylem sap of roots is an example showing effect on plant hormones. In this study, our focus is mainly anatomical changes related to biopolymer accumulation in the cell wall of root tissues. Studying adaptive responses of roots can enhance the selection of appropriate tree species for plantation sites. It is also important to identify features of root tissues unique to certain fruit tree species and to understand the changes they undergo when adapting to different soil environment. Biopolymer accumulation causes structural changes in root tissues. The Casparian strip, polyderm, periderm, *phi*-thickening, and crescent-thickening are all names of different root tissues accumulating biopolymer (such as lignin and suberin). The function of each is somehow related to protection against disease, insect attack or stressful growth conditions. The nature of accumulation may vary among fruit tree species, for example, the polyderm is a unique tissue found only in Myrtaceae, Hypericaceae, Onagraceae and Rosaceae (Esau, 1970; Fahn, 1990) and the occurrence of *phi*-thickening is consistent within a family (Gerrath et al, 2005).

Water availability in soil causes roots to undergo dramatic anatomical changes and such changes in structure effects root function so these research areas require more attention. To understand the inextricable link between root structure and its function, the task of describing microscopic structure of roots is necessary and in the case of deep rooted fruit trees, it remains largely unexplored.

Even in such a scenario, from our initial studies on fruit trees, it has become clear that phenolic and aliphatic compounds such as lignin and suberin deposition on cell walls are influenced by root age and soil water availability (Nii et al., 2004; Pan et al., 2006; Song et al., 2011). Recent studies confirm that deposition of biopolymers in the cell wall is highly site-specific (Naseer et al., 2012; Roppolo and Geldner, 2012). For example,

suberin deposition in the epidermis, exodermis and endodermis plays an important defensive role in root function and protects against stress (Peterson et al., 1978; 1982; Peterson, 1987). The radial and transverse wall of the endodermis contains suberin accumulation that forms the Casparian strip in the primary wall (Schreiber *et al.* 1994; Schreiber, 1996; Zeier and Schreiber, 1997). Roots can exhibit significant difference in the development of endodermal and exodermal layers among species (Tuladhar and Nii, 2013). How do growth conditions affect biopolymer accumulation in specific root tissues? Endodermal Casparian strip was present closer to the root tip in drought conditions (Pan et al., 2006; Song et al., 2011). Similarly, lignin accumulation in the form of phi-thickenings increased dramatically in loquat roots grown under drought conditions (Nii et al., 2004; Pan et al., 2006) and in red bayberry roots, lignin accumulation in the form of crescent-thickening in cortical cells near the endodermis, increased dramatically under drought conditions (Song et al., 2011). In the present study, variation in deposition of lignin and suberin in specific root tissues was studied in various fruit tree species according to root age and growth condition.

In chapters 1-3, Myrtaceae species of fruit trees is highlighted because in this species, a tissue named polyderm formed beyond the endodermis at a distance of 8-10 cm from the root tip, when the outer surface of the root changed from white to brown. Polyderm is a unique tissue with alternative layers of cells accumulating lignin and suberin (Esau, 1970; Fahn, 1990). The lignin accumulating cell layers were called thick-walled cells and suberin accumulating cell layers that also contained the Casparian strip were called endodermis-like cells (Nii et al., 2012). Chapter 1 highlights site-specific deposition of lignin and suberin that form a unique pattern.

In this study, we observed that biopolymers that deposit in the cell wall, causing it to

thicken, are site-specific. Lignin accumulating in the exodermis was m-shaped, however, lignin accumulating in cortex cells and two were w-shaped and this was consistent in other four species of the same family. Further study is required to understand factors that determine such site specificity in biopolymer deposition in plant tissues.

Chapter 2 provides details on observation of wax apples roots also belonging to Myrtaceae species. The number of polyderm layers that formed beyond the endodermis was influenced by soil water availability. Under flooded conditions the number of polyderm layers was fewer than roots grown in normal conditions. Decrease in polyderm layers means there were fewer cell layers with lignin and suberin indicating that the role of lignin and suberin might be greater under drought conditions.

In chapter 3, the timing of lignin accumulation in two of feijoa roots also belonging to Myrtaceae species was observed. Lignin accumulation was earlier in roots grown in drought conditions than in normal conditions. From these observations, it was confirmed that lignin accumulation in tissues increased and its accumulation became earlier when encountering drought stress.

In chapter 4, Punicaceae species is highlighted. Unlike the Myrtaceae species, alternative pattern of lignin and suberin accumulating cell layers was absent in this species. However, a periderm, several cell layers deep, existed beyond the endodermis. The cell layers forming the periderm accumulated both lignin and suberin in the same layer instead of forming separate layers for lignin and suberin. Similar observation of grapevine periderm was also conducted (Tuladhar and Nii, 2013) but lignin accumulation in periderm was unique to Punicaceae species. Chapter 4 describes details of Punicaceae roots with lignin accumulating periderm.

Finally in chapter 5, loquat roots belonging to Rosaceae species is highlighted. In

Rosaceae fruit trees, a cell-wall ingrowth caused by the deposition of lignin in the shape of a Greek letter *phi* is commonly found in cortex cells adjacent to the endodermis (Russow, 1875 as quoted by Gerrath et al., 2002). Other than endodermis and exodermis which are important for plant resistance, cortex cell wall thickenings should also be studied because it is a unique and flexible tissue found only in new root undergoing primary growth. For perennial woody fruit trees, cortex cell wall thickening may be providing mechanical support to roots but they may also be functioning as defense structures like the endodermis and exodermis.

So far, phi-thickenings have not been confirmed in seed germinated loquat roots. Development of phi-thickening in water-logged condition was studied for the first time. Our studies confirmed that phi-thickenings were present in loquat roots grown in water-logged conditions as well though the number of cortex cell layers forming phi-thickenings was fewer compared to drought conditions. Suberin-like accumulation was also observed in the intercellular space of exodermal cells and the number of cell layers with suberin deposit depended on water availability. Chapter 5 provides details of phi-thickenings observed in loquat roots.

In this way, a series of studies on root anatomy of different fruit species were conducted. In the present study, root anatomy of fruit trees were studied according to the distance from the root tip by continuously sectioning root from the tip to the basal part. However, root elongation rate is determined by soil water condition. Therefore root age cannot be determined by the distance from the root tip alone. The number of xylem vessels per pole was considered an important factor in determining root age in this study.

## References

- Armstrong, W., Brandle, R., Jackson, M. B. 1994. Mechanism of flood tolerance in plants. *Acta. Bot. Neerlandica* 43: 307-358.
- Esau, K. 1970. *Anatomy of Seed Plants*. 2nd Edition. John Wiley and Sons, New York. pp. 185.
- Fahn, A. 1990. *Plant anatomy*. 4th Edition. Pergamon Press, Oxford. pp. 33, 368.
- Gerrath, J. M., Covington, L., Doubt, J., Larson, D. 2002. Occurrence of phi thickenings is correlated with gymnosperm systematics. *Can. J. Bot.* 80: 852-860.
- Gong, D. Q., Zhu, S. J., Gu, H., Zhang, L. B., Hong, K. Q., Xie, J. H. 2013. Disease resistance of 'Zill' and 'Keitt' mango fruit to anthracnose in relation to defence enzyme activities and the content of anti-fungal substances. *J. Hort. Sci. Biotech.* 88: 243-250.
- Hawkins, S., Boudet, A. 1996. Wound-induced lignin and suberin deposition in a woody angiosperm (*Eucalyptus gunii* Hook.) histochemistry of early changes in young plants. *Protoplasma* 191: 96-104.
- Komatsu, S., Kobayashi, Y., Nishizawa, K., Nanjo, Y., Furukawa, K. 2010. Comparative proteomics analysis of differentially expressed proteins in soybean cell wall during flooding stress. *Amino Acids* 39: 1435-1449.
- Moerschbacher, B.M., Noll, U., Gorrichon, L. Reiserne, H.J. 1900. Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. *Plant Physiol.* 93: 465-470.
- Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C., Geldner, N. 2012. Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc. Natl. Acad. Sci. USA* 109: 10101-6.
- Nelson, P.E., Wilhelm, S. 1957. Some aspects of strawberry root. *Hilgardia* 26:

631-642.

Nii, N., Pan, C. X., Ogawa, Y., Cui, S. M. 2004. Anatomical features of the cell wall ingrowth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. J. Japan. Soc. Hort. Sci. 73: 411-414.

Nii, N., Ohtsuka, S., Ye, L., Song, Y. 2012. Formation of endodermis-like cells with Casparian strip and thick wall cells derived from pericycle in the roots of *Feijoa sellowiana* (Myrtaceae). J. Japan. Soc. Hort. Sci. 81: 314-319.

Pan, C. X., Nakao, Y., Nii, N. 2006. Anatomical development of *phi* thickening and the Casparian strip in loquat roots. J. Japan. Soc. Hort. Sci. 75: 445–449.

Peterson, C. A., Peterson, R. L., Robards, A.W. 1978. A correlated histochemical and ultrastructural study of the epidermis and hypodermis of onion roots. Protoplasma 96: 1-21.

Peterson, C. A., Emanuel, M. E., Wilson, C. 1982. Identification of a Casparian band in the hypodermis of onion and corn roots. Can. J. Bot. 60: 1529-1535.

Peterson, C. A. 1987. The exodermal Casparian band of onion blocks apoplastic movement of sulphate ions. J. Exp. Bot. 32: 2068-2081.

Poovaiah, B. W. 1974. Formation of callose and lignin during leaf abscission. Amer. J. Bot. 61: 829-834.

Roppolo, D., Geldner, N. 2012. Membrane and walls: who is master, who is servant? Curr.Opin. Plant. Biol. 15: 608-617.

Salvador, V.H., Lima, R.B., Dos Santos, W.D., Soares, A.R., Bohm, P.A., Marchiosi, R., Maria, L.F., Osvaldo, F.F. 2013. Cinnamic acid increases lignin production and inhibits soybean root growth. PLoS ONE 8: e69105 10.1371/ journal.pone.0069105.

Schreiber, L. 1996. Chemical composition of Casparian strips isolated from *Clivia*

*miniata* Reg. roots: Evidence for lignin. *Planta* 199: 596-601.

Schreiber, L., Breiner, H., Reiderer, M., Duggelin, M., Guggenheim, R. 1994. The Casparian strip of *Clivia miniata* Reg. roots: Isolation, fine structure and chemical nature. *Bot. Acta* 107: 353-361.

Schussler, E. E., Longstreth, D. J. 2000. Changes in cell structure during the formation of root aerenchyma in *Sagittaria lancifolia* (Alismataceae). *Amer. J. Bot.* 87: 12-19.

Shao, H. B., Chu, L. Y., Jaleel, C. A., Zhao, C. X. 2008. Water-deficit stress-induced anatomical changes in higher plants. *Comptes rendus biologiques*. 331: 215-225.

Song, Y., Ye, L., Nii, N. 2011. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. *Sci. Hort.* 129: 554–560.

Steudle, E., Peterson, C. A. 1998. How does water get through roots? *J. Exp. Bot.* 49: 775-788.

Tardieu, F., Karterji, N., Bethenod, O., Zhang, J., Davies, W. J. 1991. Maize stomatal conductance in the field: Its relationship with soil and plant water potentials, mechanical constraints and ABA concentration in the xylem sap. *Plant Cell Environ.* 14: 121-126.

Tuladhar, A., Nii, N. 2013. Anatomical changes of root thickening and shedding process of outer layers during secondary growth in grapevine roots. *Sci. Rep. Fac. Agri. Meijo Univ.* 49: 11-17. (in Japanese with English abstract).

Williamson, B. 1984. Polyderm, a barrier to infection of red raspberry buds by *Didymella applanata* and *Botrytis Cinerea*. *Ann. Bot.* 53: 83-89.

Zeier, J., Schreiber, L. 1997. Chemical composition of hypodermal and endodermal cell walls and xylem vessels isolated from *Clivia miniata*: identification of the biopolymers lignin and suberin. *Plant Physiol.* 113: 1223–1231.

## **Chapter 1**

**Formation of exclusive pattern during accumulation of ligno-suberic material in cell wall of Myrtaceae root tissues including epidermis, exodermis, endodermis and polyderm**

## 1.1 Introduction

In higher plants, the cell wall is the first compartment to be affected by stress signal (Komatsu et al., 2010, Salvador et al., 2013). Lignin and suberin that accumulate in the plant cell wall play an important protective role against fungal diseases and insect attacks (Moerschbacher et al., 1900, Nelson and Wilhelm 1957, Williamson, 1984). So far lignin and suberin accumulation has been observed in the bark (Hawkins and Boudet, 1996), the fruit (Gong et al., 2013), the leaf (Poovaiah, 1974) and roots (Steudle and Peterson, 1998) of plants.

Lignin is a polymer of phenol propane units that accumulates within the cellulose strands and toughens the cell wall (Cutter, 1970). Lignification has been described as the most important encrusting process in higher plants along with other substances like suberin, tannins etc (Fahn, 1982). It has been reported that lignin content in root tissues is affected during acclimation (Soukup et al., 2004) and by environmental stress (Nii et al., 2004, Pan et al., 2006, Song et al., 2011). Under drought stress, *phi*-thickening, a form of lignin accumulation, developed markedly in cortex cells of loquat roots (Nii et al., 2004, Pan et al., 2006). Similarly, in cortex of red bayberry roots, crescent-shaped lignin accumulation increased under drought stress conditions (Song et al. 2011). Suberin, on the other hand, is a polymer with poly (aromatic) and poly (aliphatic) domains that get impregnated in the inter microfibrillar spaces of primary cell walls to form the Casparian strip (Schreiber *et al.* 1994, Schreiber, 1996, Zeier and Schreiber 1997).

From these studies it is evident that accumulating biopolymers such as lignin and suberin in the cell wall is important for plant tissues including roots. Knowing which root tissues accumulate lignin or suberin may provide further insight to understand their physiological role in plant protection.

Myrtaceae is one of the few plant families (other than Hypericaceae, Onagraceae and Rosaceae) that develop a special type of protective tissue called polyderm in its roots that consist of alternating suberized and non-suberized cell layers (Esau, 1970; Fahn, 1982). In our previous paper (Nii et al., 2012) we explained how polyderm layers form due to cell division of the pericycle in feijoa (Myrtaceae) roots. One layer of pericycle divided into three layers. Lignin accumulated in the outermost layer and we called that layer thick-walled cell layer. Suberin accumulated in the middle layer forming the Casparian strip so we called that layer endodermis-like cell layer and the innermost layer retained the pericyclic characters that helped it divide further into a new set of three layers. During this observation, our attention was drawn to biopolymers that accumulated in polyderm tissues.

In this investigation we attempted to locate the accumulation of such phenolic substances (mainly lignin and suberin) in various root tissues such as 1) epidermis (ep), 2) exodermis (ex), 3) cortex (co), 4) endodermis (en), 5) endodermis-like cells (el), and 6) thick-walled cells (twc) in five species belonging to Myrtaceae family with special focus on feijoa. Since chemical analysis of these substances was not conducted, we have called it ligno-suberic material as phrased by Roppolo et al. (2011).

*Feijoa sellowiana* is a subtropical species of Myrtaceae family native to South Brazil with secondary dispersion in Uruguay (Thorp and Bielecki, 2002). As a newly rising species with ornamental and medicinal properties that also bear edible fruits, feijoa shows great potential in foods, drugs and cosmetics (Hardy and Michael, 1970, Kolesnik et al., 1991). In contrast to other plant structures (O' Brien, 1994, Canhoto et al., 1996, O' Brien et al., 1996, Kitayama et al., 1997, Zhang et al., 2009, Zhang et al., 2011) anatomical studies on root structures of Myrtaceae are very limited, especially those

concerning biopolymer deposition at the polyderm.

## 1.2 Materials and Methods

Five species of Myrtaceae family namely: 1) rose apple (*Syzygium jambos*), 2) strawberry guava (*Psidium cattleianum* Sabine), 3) wax apple (*Syzygium samarangense*), 4) guava (*Psidium Guajava* L), 5) feijoa (*Feijoa sellowiana*) were used for the present investigation. Three-year-old trees were grown in baskets (diameter and depth: 300 mm each, stitch size: 30 mm) covered with non-woven fabric and filled with sandy soil in a greenhouse from spring to summer of 2013 under normal conditions. The plants were watered when the upper surface of the soil became dry. To collect roots, plants were removed from the baskets. New white roots were collected from the root tip to the basal zone. Hand-sections of roots were prepared with a new stainless steel razor blade. Serial transverse sections, commencing at approximately 5 mm from the root tip toward the basal portion of the root, were cut at 10 mm intervals to examine changes in the structural development as roots matured.

Root tissues were examined under fluorescent microscopy (excitation wavelength 365nm) staining with or without several different solutions, including a series of berberine hemisulfate-aniline blue (Brundrett et al. 1988), or phloroglucinol-HCL for lignin (Jensen 1962). Lux et al. (2005) reported a novel method for simultaneously clearing and staining tissue in which the fluorochrome 0.1% berberine hemisulfate was dissolved in a clearing mixture consisting of pure lactic acid saturated with chloral hydrate. We used this method and incubated the root sections in 0.1% berberine hemisulfate solution at 40°C for 30 minutes. The sections were then counterstained with 0.5% aniline blue (dissolved in H<sub>2</sub>O) at room temperature for 30 minutes followed by

saffranin O for 1 minute. All specimens were then placed on a drop of H<sub>2</sub>O on a slide glass and observed under ultraviolet light using fluorescent microscopy.

Sections of the same root samples used for hand sectioning were fixed in 3 % glutaraldehyde (TAAB Laboratories, England, UK) in 0.1 M cacodylate buffer, (Sigma Aldrich, Japan, pH 7.2) and stored at 4°C to examine the anatomical features of cell development. The samples were then dehydrated through a graded ethanol series and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany). Semi-ultra thin sections (1.5 µm) were cut using a glass knife then stained with methylene blue for histological examination under a BX 60 optical microscope (Olympus, Tokyo, Japan).

Although root age can be determined by surface color (i.e., white roots are young and brown roots are old) and by the distance from the root tip, we considered the degree of xylem differentiation, i.e., the number of xylem vessels per pole as well. When estimating root maturity, taking xylem differentiation into account is considered more accurate than root color or distance from the root tip. For observation, more than twenty roots were sectioned per species but a representative root was selected for report since the basic anatomical features were similar among all root samples. A detailed study of feijoa roots was conducted then similar traits were examined in other four species of Myrtaceae.

### **1.3 Results**

Firstly, three root tissues namely the epidermis (Fig. 1B, ep), the cortex cells surrounding the endodermis (Fig. 1A, co) and the thick-walled cells existing beyond the endodermis (Fig.1 A, twc) were examined with or without staining. The term ‘thick-walled cell’ is used because the cell wall of this cell gets impregnated with phenolic substances on one side.

When stained with berberine hemisulfate - aniline blue - safranin O and observed under a fluorescent microscope, the epidermis (Fig. 1B, ep) and thick-walled cells (Fig. 1A, twc) turned reddish orange color. When stained with phloroglucinol and observed under an optical microscope, the epidermis cells (Fig. 1D, ep) and the thick-walled cells (Fig. 1C, twc) turned pinkish-red showing that lignin was present in these tissues. When unstained root samples were observed under fluorescent microscope, the epidermis (e.g. Fig. 4F, ep) and the thick-walled cells (e.g. Fig. 4D, twc) emitted the bluish-white light indicating that phenolic substances other than lignin could be present in these tissues.

A different accumulation pattern was noted at the epidermis and the thick-walled cells (Figs. 1A, B, small arrows). At the epidermis, the lignin accumulation appeared to be “open” centripetally resembling the letter “m” (Figs. 1B,D, ep) whereas at the thick-walled cells it appeared to be “closed” centripetally resembling the letter “w” (Figs. 1A,C, twc).

When root samples embedded in Technovit 7100 resin were stained with methylene blue and observed under an optical microscope, accumulation of phenolic substances was observed on only one side of the cell wall “closed” centripetally to the central stele (Figs. 2A,B, arrowhead). Both cortical cells surrounding the endodermis (Fig. 2A, arrowhead) and the thick-walled cells (Fig. 2B, arrowhead) accumulated, mainly lignin. The thick-walled cells formed at two areas, initially between the original endodermis and endodermis-like cell layer (Fig. 3 A, twc) and successively between every endodermis-like cell layer at the polyderm (Figs. 3 B, C, twc). It must be highlighted here that the term ‘endodermis-like cell’ layer is used because it forms beyond the original endodermis and bears the Casparian strip, with suberized cell walls resembling the endodermis. The formation of endodermis-like cell layers is a result of secondary growth

of roots.

Secondly, other three root tissues namely the exodermis, the endodermis and the endodermis-like cells were examined. When root samples stained with berberine hemisulfate - aniline blue - safranin O were observed under a fluorescent microscope, the exodermis (Fig. 1B, ex), the endodermis (Fig. 1A, en) and the endodermis-like cells (Fig. 1A, el) existing beyond the endodermis appeared bluish- white. When unstained root samples were observed under fluorescent microscope, these tissues still emitted the same bluish-white color (Fig. 4) implying phenolic substances most likely to be suberin accumulated in these tissues.

Suberization and the Casparian strip formation in the endodermis and endodermis-like cells occurred as roots matured. In younger roots with about 8-10 vessels at each xylem pole, suberin-like accumulation was observed on the entire cell wall at the exodermis (Fig. 4E, ex) whereas at the endodermis, Casparian strips appeared to be small line-like (Fig. 4A, en) initially then biopolymers, including suberin, accumulated in the entire cell wall of all endodermis cells (Fig. 4B, en). In the endodermis-like layers, suberic substances accumulated on only one side of the cell wall opposite to the stele appearing to be “open” centripetally resembling the letter “m” (Fig. 3C, large arrow; Fig. 4D).

In tissues closer to the soil surface, such as epidermis and exodermis, accumulation of biopolymers were aligned (Fig.1B, small arrow and large arrow). In other words, both appeared to be “open” centripetally resembling the letter “m”. In tissues beyond the endodermis, biopolymers mostly suberin, accumulation in the endodermis-like cells appeared to be “open” centripetally resembling the letter “m” whereas accumulation in the thick-walled cells appeared to be “closed” centripetally resembling the letter “w.”

(Figs.3 B, C, twc, el). Like feijoa, this pattern of biopolymer accumulation was common in all four species of Myrtaceae (Fig. 5).

#### **1.4 Discussion**

From the present study, a common pattern in biopolymer accumulation was detected in cross sections of all examined Myrtaceae roots (Fig.5). Biopolymer accumulation was observed in specific area of the cell wall in specific tissues. Since chemical analysis was not conducted in this study, it was not possible to distinguish whether biopolymers accumulating in tissues were only lignin or suberin and since their chemical composition differs their physiological role may vary. Bernards (2002) highlighted that suberized tissues possess a specialized cell wall modification consisting of poly (aliphatic) and poly (phenolic) domains which are spatially distinct but coexist in suberized cells. He further mentioned that the phenolic monomer profiles for lignified and suberized tissues are different and tissue specific.

In the examined roots, we concluded that biopolymer accumulating in epidermis and thick-walled cells was mostly lignin because these tissues turned red when stained with phloroglucinol (Figs. 1C, D). Furthermore, in roots embedded in Technovit, noticeable “w” shaped cell wall thickenings were observed (Fig. 2).

Earlier studies on biopolymers have examined the amount of suberin and lignin accumulation in root tissues. According to Bernards (2002), in general there is enough evidence supporting the hypothesis that suberized tissues contain a significant amount of hydroxycinnamic acids and a relatively smaller amount of monolignols than expected for a lignified tissue. He summarized the results of these analyses as follows: as one moves from the stele to the epidermis of roots, there is an increasing amount of

hydroxycinnamic acid in the poly (phenolic) components of the cell walls, ranging from essentially 100% monolignol in the stele (as expected for lignified walls of the vascular tissues) to a mixture of monolignol and hydroxycinnamic acid in the epidermis. However, the actual area of accumulation in the cell wall of root tissues has not gained much attention.

This anatomical study has its limitations because it is not sufficient to determine the chemical nature of substances accumulating in the polyderm. Esau (1970) has defined polyderm as a tissue composed of alternating layers of suberized and non-suberized cells. What substances accumulate in the non-suberized cells of polyderm is not mentioned. This is the layer that is termed as thick-walled cell layer in this paper, specifically because accumulation of what seems to be dominantly lignin, caused noticeable “w” shaped swelling/ thickening in its cell wall. This study points out the actual area of the cell wall where biopolymers get accumulated in different root tissues.

Biopolymers impregnate plant cell wall to establish a paracellular diffusion barrier. However, the mechanism of generating this tight junction remains unclear in molecular terms (Naseer et al. 2012). Perhaps, positioning of biopolymer in the cell wall is crucial for selective uptake of nutrients and pathogen exclusion because it could affect apoplastic pathways of water.

The present study alone is not enough to explain why ligno-suberic materials accumulate on opposite sides of the cell wall in polyderm tissues. Nevertheless, it has provided details of a distinct lay out pattern of phenolic substances deposited in polyderm, a tissue unique to roots belonging to only a small number of plant families including Myrtaceae. Although the significance of such accumulation pattern is unknown, it is clear that certain factor/s regulates the deposit of biopolymers in this manner and it is

beneficial to the plant.

The presence of lignin in thick-walled cells between every endodermis-like cell layer and the existence of multiple layers of Casparian strips in the endodermis-like layers, in addition to the original endodermis, could be functioning as multiple filters to enhance root function even under unfavorable growth conditions. Could the biopolymer accumulation in the polyderm be affected by stress conditions? Our previous results have shown that in wax apple (*Syzygium samarangense*) roots belonging to Myrtaceae, the number of suberized endodermis-like cell layers increased up to 5 layers and thick-walled cells with lignin increased up to 10 layers at some areas of the root circumference under drought stress conditions (unpublished data).

Lastly, this anatomical study has broadened our knowledge concerning polyderm bearing roots of Myrtaceae at cell level. It is interesting to question - what factors might limit biopolymers from accumulating in the entire cell walls of polyderm and how plants can benefit from this alternating pattern of biopolymer deposition.

## **1.5 Summary**

Details on localized deposition of biopolymers in cell wall have rarely been studied. Anatomical observation is important to understand tissue-specific accumulation of biopolymers and such knowledge can further clarify tissue-function. Myrtaceae roots possess alternating suberized and non-suberized cell layers known as polyderm that exist beyond the endodermis. It is important to understand firstly, where biopolymers accumulate in this tissue and secondly what mechanisms control cell wall modification. This study aims to study areas of biopolymer deposition in Myrtaceae root tissues.

Root specimens were sectioned freehand or with an ultramicrotome after embedding

in Technovit 7100 resin. Root sections were stained with berberine hemisulfate-aniline blue-safranin O series or just phloroglucinol and observed under a fluorescent or optical microscope.

Different biopolymers accumulated alternatively on opposite sides of the cell wall in polyderm. In non-suberized tissues, lignin accumulation was dominant and its accumulation appeared to be “closed” centripetally resembling the letter “w.” In suberized tissues, it resembled the letter “m”. This tissue-specific accumulation pattern was common in all five Myrtaceae species.

What factors could control and regulate such patterned and tissue-specific accumulation of biopolymers? Could this accumulation pattern itself be a contributing factor to its protective role? Discovery of this pattern, specific to Myrtaceae root polyderm, triggers more investigations on the effect of biotic and abiotic stress on biopolymer accumulation in it.

## **1.6 References**

- Bernards, M.A. 2002. Demystifying suberin. *Can. J. Bot.* 80: 227-240.
- Brundrett, M.C., Enstone, D.E., Peterson, C.A. 1988. A berberine-aniline blue fluorescent staining procedure for suberin, lignin and callose in plant tissue. *Protoplasma* 146: 133–142.
- Canhoto, J.M., Mesquita, J.F., Cruz, G.S. 1996. Ultrastructural changes in cotyledons of pineapple guava (Myrtaceae) during somatic embryogenesis. *Ann. Bot.* 78: 513-521.
- Cutter, E.G. 1970. *Plant Anatomy: Experiment and Interpretation*. Edward Arnold, London. pp. 39
- Esau, K. 1970. *Anatomy of Seed Plants* 2nd Edition. John Wiley and Sons, New York.

pp. 185.

Fahn, A. 1990. Plant anatomy 4th Edition. Pergamon Press, Oxford. pp. 33, 368

Gong, D.Q., Zhu, S.J., Gu, H., Zhang, L.B., Hong, K.Q., Xie, J. H. 2013. Disease resistance of 'Zill' and 'Keitt' mango fruit to anthracnose in relation to defence enzyme activities and the content of anti-fungal substances. J. Hort. Sci. Biotech. 88: 243-250.

Hardy, P.J., Michael, B.J. 1970. Volatile components of feijoa fruits. Phytochem. 9: 1355-1357.

Hawkins, S., Boudet, A. 1996. Wound-induced lignin and suberin deposition in a woody angiosperm (*Eucalyptus gunii* Hook.) histochemistry of early changes in young plants. Protoplasma 191: 96-104.

Jensen, W.A. 1962. Botanical Histochemistry - principles and practice. University of California, Berkeley. W.H. Freeman and Company, pp. 205.

Kitayama, K., Pattison, R., Cordell, S., Webb, D., Dombois, D.M. 1997. Ecological and genetic implications of foliar polymorphism in *Metrosideros polymorpha* Gaud. (Myrtaceae) in a habitat matrix on Mauna Loa, Hawaii. Ann. Bot. 80:491-497.

Kolesnik, A.A., Golubev, V.N., Gadzhieva, A.A. 1991. Lipids of the fruit of *Feijoa sellowiana*. Chem. Nat. Compd. 27: 404-407.

Komatsu, S., Kobayashi, Y., Nishizawa, K., Nanjo, Y., Furukawa, K. 2010. Comparative proteomics analysis of differentially expressed proteins in soybean cell wall during flooding stress. Amino Acids 39: 1435-1449.

Lux, A., Morita, S., Abe, J., Ito, K. 2005. An improved method for clearing and staining free-hand sections and whole-mount samples. Ann. Bot. 96: 986-996.

Moerschbacher, B.M., Noll, U., Gorrichon, L., Reiserne, H.J. 1900. Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. Plant Physiol. 93:

465-470.

Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C., Geldner, N. 2012. Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. Proc. Natl. Acad. Sci. USA 109: 10101-6.

Nelson, P.E., Wilhelm, S. 1957. Some aspects of strawberry root. Hilgardia 26: 631-642.

Nii, N., Ohtsuka, S., Ye, L., Song, Y. 2012. Formation of endodermis-like cells with Casparian strip and thick wall cells derived from pericycle in the roots of *Feijoa sellowiana* (Myrtaceae). J. Japan. Soc. Hort. Sci. 81: 314-319.

Nii, N., Pan, C.X., Ogawa, Y., Cui, S.M. 2004. Anatomical features of the cell in-growth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. J. Japan. Soc. Hort. Sci. 73: 411-414.

O'Brien, S.P. 1994. Pistil structure and pollen tube pathways in *Leptospermum myrsinoides* and *L. continentale* (Myrtaceae). Ann. Bot. 73: 225-230.

O'Brien, S.P., Loveys, B.R., Grant, W.J.R. 1996. Ultrastructure and function of floral nectaries of *Chamelaucium uncinatum* (Myrtaceae). Ann. Bot. 78: 189-196.

Pan, C.X., Nakao, Y., Nii, N. 2006. Anatomical development of *phi* thickening and the Casparian strip in loquat roots. J. Japan. Soc. Hort. Sci. 75: 445-449.

Poovaiah, B.W. 1974. Formation of callose and lignin during leaf abscission. Amer. J. Bot. 61: 829-834.

Roppolo, D., Rybel, B. D., Tendon, V.D., Pfister, A., Alassimone, J., Vermeer, J., Yamazaki, M., Stierhof, Y., Beeckman, T., Geldner, N. 2011. A novel protein family mediates Casparian strip formation in the endodermis. Nature 473: 380-383.

Salvador, V.H., Lima, R.B., Dos Santos, W.D., Soares, A.R., Bohm, P.A., Marchiosi, R.,

- Maria, L.F., Osvaldo, F.F. 2013. Cinnamic acid increases lignin production and inhibits soybean root growth. PLoS ONE 8: e69105 10.1371/journal.pone.0069105.
- Schreiber, L. 1996. Chemical composition of Casparian strips isolated from *Clivia miniata* Reg. roots: Evidence for lignin. Planta 199: 596-601.
- Schreiber, L., Breiner, H., Reiderer, M., Duggelin, M., Guggenheim, R. 1994. The Casparian strip of *Clivia miniata* Reg. roots: Isolation, fine structure and chemical nature. Bot. Acta 107: 353-361.
- Song, Y., Ye, L., Nii, N. 2011. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Hort. 129: 554–560.
- Soukup, A., Mala, J., Hrubcova, M., Kalal, J., Votrubova, O., Cvikrova, M. 2004. Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry-tree plantlets during acclimation. Bio. Planta. 48: 481-489.
- Steudle, E., Peterson, C.A. 1998. How does water get through roots? J. Exp. Bot. 49: 775-788.
- Thorp G, Bielecki R 2002 Feijoas: Origins, Cultivation and Uses, Hort Research, New Zealand, pp. 87.
- Williamson, B. 1984. Polyderm, a barrier to infection of red raspberry buds by *Didymella applanata* and *Botrytis Cinerea*. Ann. Bot. 53: 83-89.
- Zeier, J., Schreiber, L. 1997. Chemical composition of hypodermal and endodermal cell walls and xylem vessels isolated from *Clivia miniata*: Identification of biopolymers lignin and suberin. Plant Physiol. 113:1223-1231.
- Zhang, M., Tang, H.R., Wang, D., Ren, S.X., Liu, R.D. 2009. A study of rooting characteristics and anatomical structure of feijoa cuttings. Agri. Jour. 4: 86-90.

Zhang, M., Tang, H.R., Wang, D., Ren, S.X., Fan, L.Z. 2011. Study on growth characteristics of young feijoa trees in different propagation methods. Bot. Res. J. 4: 26-28.

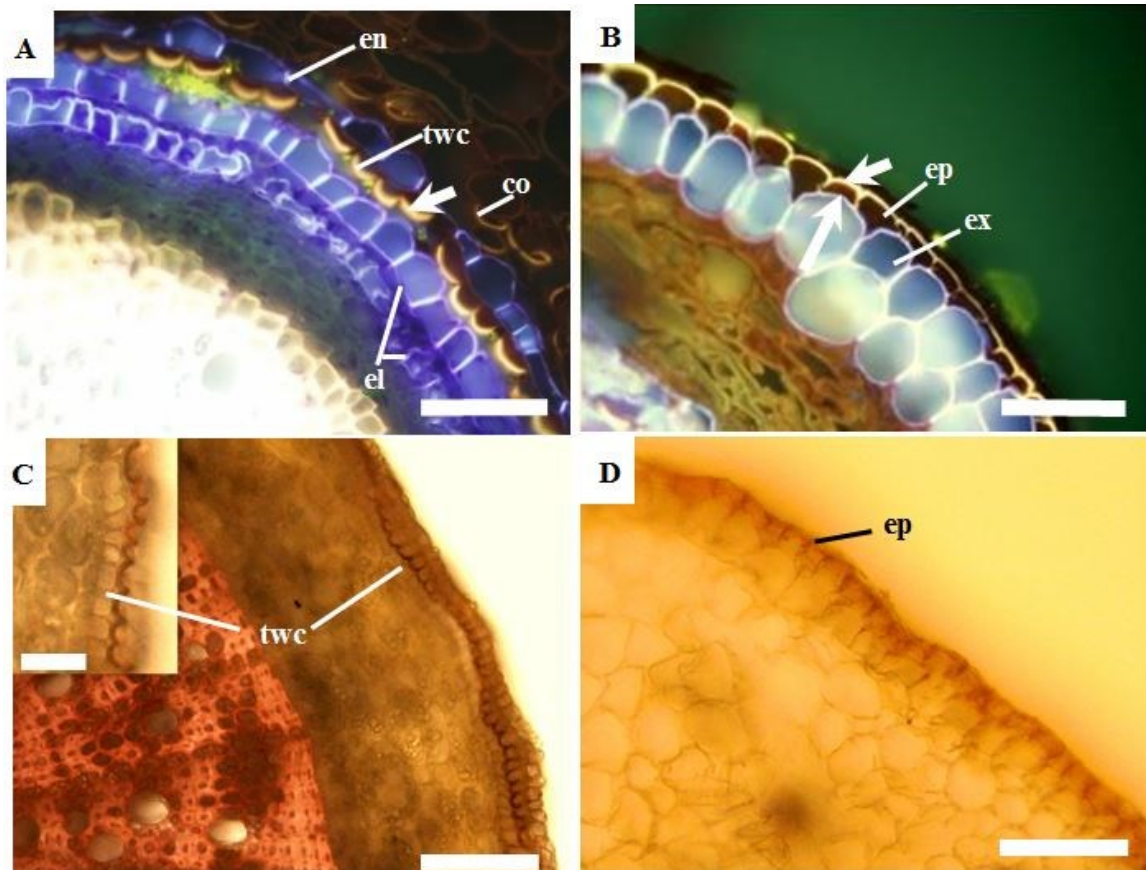


Fig.1-1: Difference in lignin accumulating area in the cell wall of root tissues of feijoa roots. Panels A and B show berberine hemisulfate-aniline blue-safranin O stained root samples in which lignin encrusted tissues turned reddish orange under the fluorescent microscope. Panels C and D show phloroglucinol stained root samples in which lignin encrusted tissues turned pinkish-red when observed under optical microscope. In thick-walled cells (twc in Panel A and C) lignin accumulation appeared w-shaped and “closed” centripetally. At the epidermis, (small arrow in Panel B and ep in Panel D) it was m-shaped and “open” centripetally. ep: epidermis; ex: exodermis; en: endodermis; co: cortex; twc: thick-walled cell; el: endodermis-like cell, small arrow: lignin accumulation, large arrow: suberin accumulation. Scale bars = 50  $\mu$ m (Panels A, B and enlarged section within Panel C), 100  $\mu$ m (Panels C, D ).

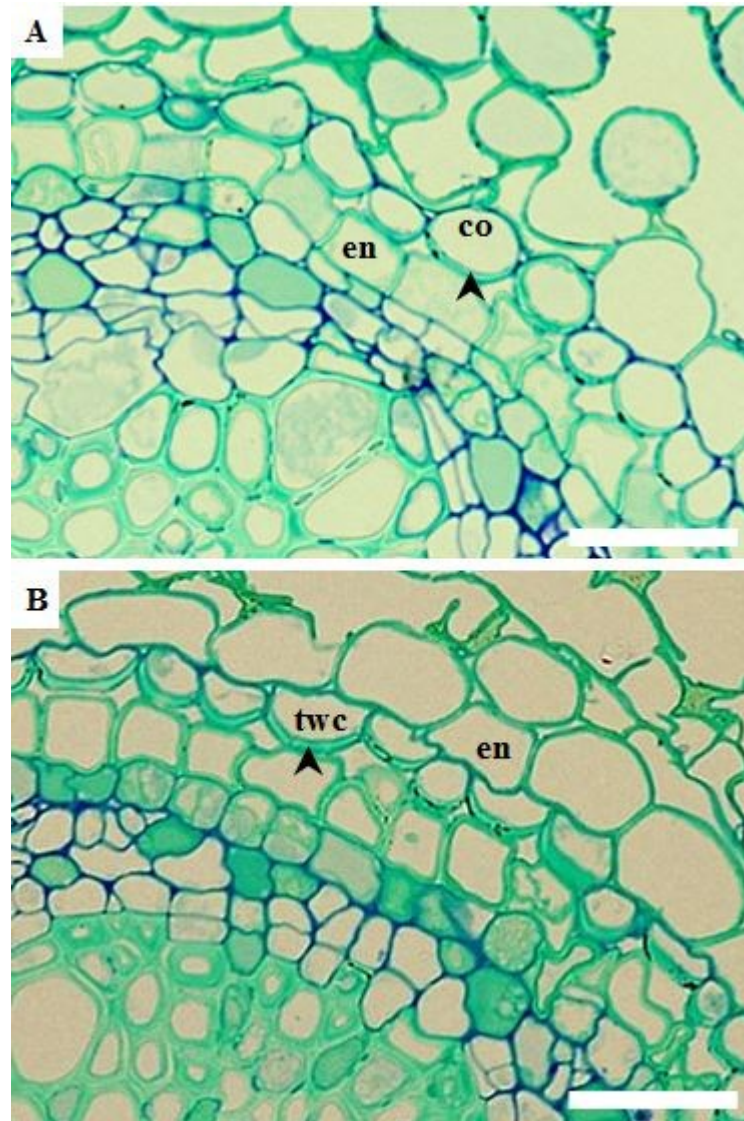


Fig.1-2: Observation of accumulation of biopolymers, mostly lignin, in feijoa root sample embedded in Technovit 7100 resin after staining with methylene blue. Cross sections (1.5 $\mu$ m) were examined under optical microscope. Arrowheads show swelling caused by lignin accumulation in cortex in panel A and in thick-walled cells in panel B. en: endodermis; co: cortex; twc: thick-walled cell. Scale bars = 50  $\mu$ m.

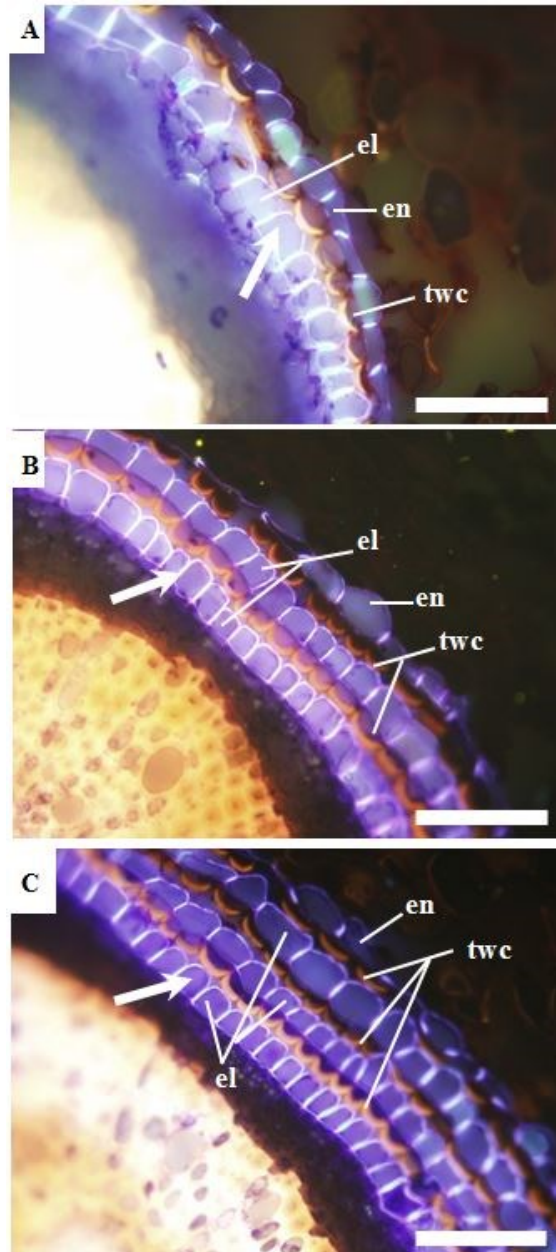


Fig.1-3: Increase in polyderm layers beyond the endodermis in cross sections of feijoa roots. Panel A shows lignified thick-walled cells formation between the original endodermis and endodermis-like cell. Panels B and C show lignified thick-walled cells formation between every suberized endodermis-like cell layer with Casparian strip. en: endodermis; el: endodermis-like cell; twc: thick-walled cell. The large arrow shows suberin accumulation which is m-shaped and “open” centripetally. Distance from the root tip is 80 mm (Panel A), 100 mm (Panel B), 120 mm (Panel C). Scale bars = 50 μm.

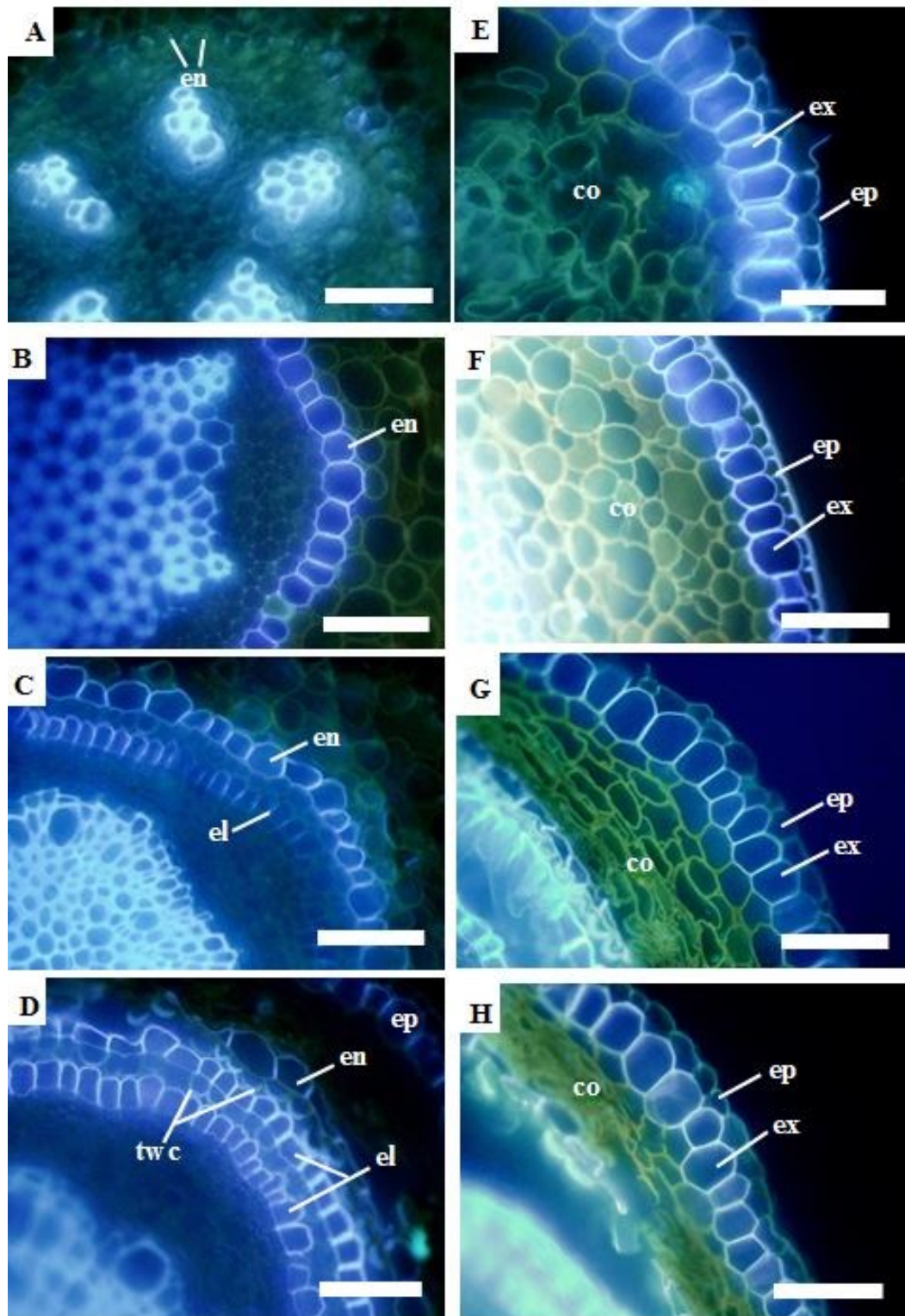


Fig.1-4: Suberin accumulation in feijoa root tissues according to distance from the root tip. Unstained cross sections were observed under fluorescent microscope. Distance from the root tip is 10 mm (Panels A, E), 40 mm (Panels B, F), 60 mm (Panels C, G),

80 mm (Panels D, H). Left panels show suberization with Casparian strip at endodermis (en) and endodermis-like cells (el). Right panels show suberization with Casparian strip at exodermis (ex). The bluish white autofluorescence in lignin encrusted tissues such as the epidermis (ep) and the thick-walled cells (twc) indicating that these tissues accumulate substances other than lignin. The compression in cortex (co) layers can be observed in Panels E-H. Scale bars = 50  $\mu$ m.

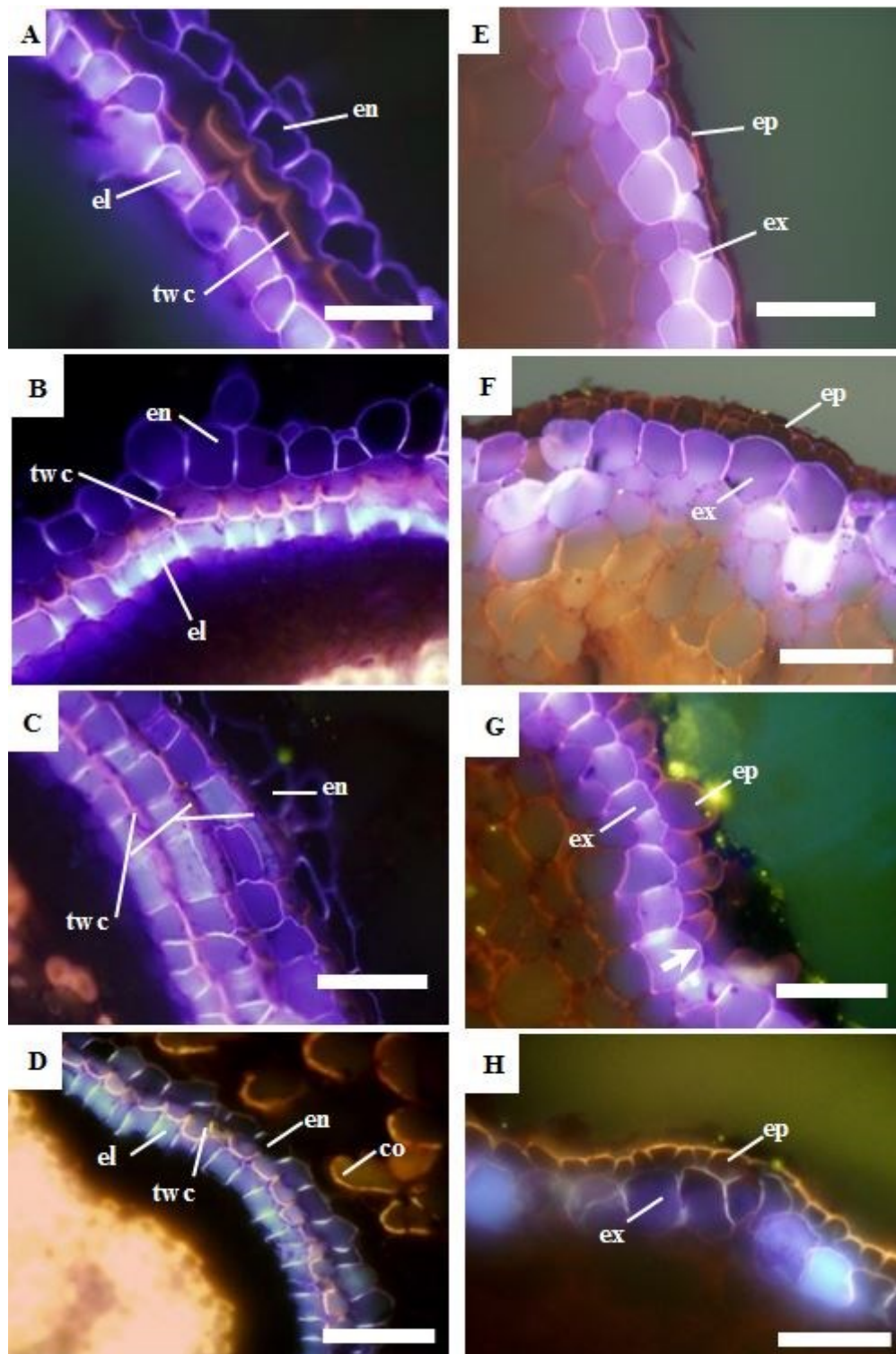


Fig. 1-5: Common traits of lignin and suberin accumulation found in stained cross-sections of four Myrtaceae species (A, E: *Syzygium jambos*, B, F: *Psidium*

*cattleianum*, C, G: *Syzygium Samarangense*, D, H: *Psidium Guajava* L). Left panels (A-D) show w-shaped, lignified thick-walled cells (twc) forming between suberized endodermis-like layers (el) in the polyderm. Right panels (E-H) show m-shaped lignin and suberin accumulation in epidermis (ep) and exodermis (ex). Lignified tissues were appeared reddish orange and suberized tissues appeared bluish white when observed under fluorescent microscope. co: lignin accumulation in cortex cell wall. Scale bars = 50µm.

## **Chapter 2**

**Anatomical study on wax apple (*Syzygium samarangense*) roots under long-term water-logged conditions**

## 2.1 Introduction

Wax apple (*Syzygium samarangense*), also called wax jambu or Java apple, is native to a wide range of areas from Malaysia to Andaman and Nicobar Islands of India (Morton, 1987). For successful off-season production of wax apples, techniques related to roots, like root pruning and short-term flooding are being used in Taiwan to induce flowering (Shu et al., 2007). Water-logging creates a hypoxic soil environment so it can directly affect root functionality. However, studies on root response to water-logging, especially long-term water-logging which is more likely to occur in the tropics due to seasonal heavy rains, is important but rare. To understand the effects of long-term water-logging on root development is fundamental for tropical fruit tree cultivation. Plants vary considerably in their ability to tolerate water-logged soil (Kozlowski, 1984). Specific flooding responses of a wide range of fruit crops have been reviewed by Rowe and Beardsell (1973) and Schaffer et al. (1992). Hsu et al. (1999) have reported that in wax apples, the activity of nitrate reductase in roots of water-logged trees was not found to increase, as it did in other plants, instead it decreased significantly. Though such biochemical studies have been reported, anatomical study is required to observe structural changes occurring in root tissues. Particularly, wax apple roots bear a special type of protective tissue called polyderm composed of alternating suberized and non-suberized layers, common in only a few other plant families like Hypericaceae, Onagraceae and Rosaceae (Esau, 1970; Fahn, 1982). Biopolymers like lignin and suberin are cell wall components and specialized plant cells often locally modify their cell walls by accumulating them as part of a developmental program or when challenged by environmental conditions (Roppolo and Geldner, 2012). In higher plants, the cell wall is the first compartment to be affected by stress signal (Komatsu et al., 2010, Salvador et al.,

2013).

The effect of soil water availability on polyderm tissue is an unexplored topic. According to our previous observation in roots of loquat (*Eriobotrya japonica* Lindl.), *phi*-thickening, a form of lignin accumulation in the cell wall, developed dramatically in loquat root cortex under drought condition (Nii et al., 2004; Pan et al., 2006). In the case of red bayberry (*Myrica rubra* Sieb. et Zucc.), lignin accumulation that were crescent-shaped developed noticeably in the root cortex under normal and drought condition than in water-logged condition (Song et al., 2011). Loquat and red bayberry both did not bear polyderm in their roots, however, in both cases, lignin accumulation increased in root tissues under drought stress. Can water-logging alter the accumulation of biopolymers like lignin and suberin? We hypothesized that water-logging will have adverse effect than drought, which means a decrease in lignin accumulation in root tissues. However, water-logging also means plants have continuous access to water. It was acknowledged that side-effects like depletion of oxygen in water-logged soil could alter the soil structure itself and was considered part of the treatment. In this study, water-logging period was prolonged to examine root response of polyderm-bearing plants particularly because cell wall modification in polyderm layers was thought to be effected by water-logging. So far, roots of many Myrtaceae such as rose apple (*Syzygium jambos*), strawberry guava (*Psidium cattleianum*), guava (*Psidium Guavaja*), feijoa (*Feijoa sellowiana*) were also observed but the most number of polyderm layers was observed in wax apples and so it was chosen for this study.

This anatomical study aims to observe and record detailed changes in wax apple root tissues under water-logged conditions especially during growth of young white roots and secondary growth of older brown roots.

## 2.2 Materials and Methods

Three-year-old wax apple (cultivar 'Pink' originating in Taiwan) trees propagated from cuttings were grown in plastic pots (diameter 300 mm, depth 300 mm) covered with nonwoven fabric and filled with sandy soil. The young trees were grown under water-logged and normal treatments in a greenhouse located at Meijo University, Nagoya, Japan. Five pots were allotted per treatment batch. The water-logged pots were watered every day until it saturated the soil in pots, leaked out of pot holes and collected in plastic dishes placed at the bottom of the pots. Water in these dishes was maintained at a depth of several centimeters. Pots treated normally were watered only when the upper surface of the soil became dry and no dish was placed under these pots. According to the weather, it took 2-4 days for the upper surface to be dry. The magnitude of the stress was checked several times during treatment by assessing the water potential using a pressure chamber (Plant Moisture Stress Measurements by PMS Instrument Company, Oregon, U.S.A). A leaf attached to petiole was collected from each plant in air-tight plastic bag and delivered to the lab in an icebox for immediate measurement of water potential before noon. The value of the pressure chamber was maintained at approximately  $-0.5$  MPa for water-logged plants. For normally treated plants, the value of the pressure chamber was between  $-1.0$  MPa to  $-1.5$  MPa just before watering the pots. The treatment period was about six months from early May to mid-October in 2013.

To collect roots, plants were gently removed from the baskets without disturbing the soil that was still intact with the root mass. New white roots were collected from the tip to the basal zone. Then they were hand-sectioned using a stainless steel razor blade immediately after collection. Serial transverse sections, commencing at about 5 mm from the root tip toward the basal portion of the root, were cut at 10 mm intervals to

examine changes in structural development as the root aged.

Root specimens were then examined under fluorescent microscopy (excitation wavelength 365 nm) with or without staining. Several different staining solutions, including berberine hemisulfate-aniline blue for suberin lamellae with Casparian strips (Brundrett et al., 1988), saffranin O or phloroglucinol-HCL for lignin (Jensen 1962) were used. Lux et al. (2005) reported a new method for simultaneously clearing and staining tissue, in which the fluorochrome 0.1% berberine hemisulfate was dissolved in a clearing mixture consisting of pure lactic acid saturated with chloral hydrate. We used this method, and incubated the root sections in 0.1% berberine hemisulfate solution at 40°C for 30 minutes. The sections were counterstained with 0.5% aniline blue (dissolved in water) at room temperature for 30 minutes followed by saffranin O for 1 minute. All specimens were then placed on a drop of H<sub>2</sub>O on a slide glass then observed under ultraviolet light using fluorescent microscopy.

To examine the anatomical features in detail, the root tissues remaining after hand sectioning were fixed in 3% glutaraldehyde (TAAB, Laboratories, England, UK) in 0.1 M cacodylate buffer (Sigma Aldrich, Japan), pH 7.2 and stored at 4°C. The samples were then dehydrated through a graded ethanol series and embedded in Technovit 7100 resin (Kulzer Heraeus GmbH, Wehrheim, Germany). Semi-ultrathin sections (1.5 µm), cut using a glass knife, were stained with methylene blue for histological examination under a BX60 optical/ultraviolet microscope (Olympus, Tokyo, Japan).

Although the root age can be determined by surface color (i.e., white roots are young and brown roots are old) and by the distance from root tip, we determined root age by assessing the degree of xylem differentiation, i.e., the number of xylem vessels per pole as well. To estimate root maturity, this method is considered more accurate than root

color or the distance from the root tip. More than twenty roots were sectioned and observed in each treatment. Then a representative root was selected for report since the basic anatomical features were similar among root samples.

### **2.3 Results**

In both water-logged and normal treatments, the external appearance of the root was creamy white near the root tip, but at the basal portion, the color gradually changed to light brown. In cross sections of younger roots with about 8-12 xylem vessels per pole (about 10 mm behind the root tip), some cells remained non-suberized at the passage areas of the endodermis (Figs. 1 A, C). Lignin-like accumulation was observed in inner cortical cells in both treatments. However, they were fewer in water-logged than in normally grown roots (Fig. 1).

Aerenchyma (ae) developed in the cortex area under both treatments (Fig.2). The size and number of aerenchyma increased in water-logged roots (Figs. 2C, D) than normal (Figs. 2 A, B). Aerenchyma formed due to collapse of cortical cell wall.

When root color changed from white to brown (about 100 mm behind the root), thick-walled cells and endodermis-like cell layers, with Casparian strip and suberin accumulation, developed beyond the endodermis (Figs. 3B, F). As the central stele continued to enlarge further (about 120 mm behind the root tip), up to 5 layers of endodermis-like cells were observed in normally treated roots (Fig. 3D) whereas in water-logged roots, the most number of endodermis-like cell layers observed was 3 (Table 1). In water-logged roots, polyderm layers were fewer than normal batches (Table 1).

It was noted that the tangential width of the endodermis-like cells decreased

progressively from the first to the fifth layer (Fig. 3D). The width of the first layer of endodermis-like cells was  $21.0 \pm 2.36 \mu\text{m}$  (mean  $\pm$  SD,  $n = 6$ ) and the fifth layer was  $11.8 \pm 3.76 \mu\text{m}$  ( $n = 7$ ). The outer endodermis-like cell layers expanded more than inner endodermis-like cell layers. They expanded gradually to accommodate the growing stele as outermost layers sloughed off.

The thick-walled cell layers, existing between endodermis-like cells, turned red when stained with phloroglucinol just like the xylem when observed under optical microscope (Fig. 3C enlarged photo). It turned reddish orange when stained with berberine hemisulfate-anniline blue and saffranin O and observed under the fluorescent microscope. Lignin-like deposit was observed in their cell walls but they did not deposit in the entire cell wall. Lignin accumulation was closed centripetally resembling the letter “u” (Fig. 3B, twc, enlarged photo). We called these layers thick-walled cell layers. They formed immediately adjacent to the endodermis and between every endodermis-like cell layer (Fig. 3).

From these observations, it was clear that lignin-like accumulation and polyderm layers were fewer in water-logged roots but aerenchyma developed more in water-logged than in normally treated roots.

## **2.4 Discussion**

This anatomical investigation was conducted to record structural changes in young and older root tissues of wax apples due to prolonged water-logging. Exposure to excess stagnant water in the soil for extended periods can alter soil structure by causing oxygen deficiency and this factor was taken into account as part of the treatment. Water-logging can affect the functionality of woody fruit tree roots that hardly get

transplanted once they are planted. Result from this anatomical study sheds light on tissue-specific cell wall modification after encountering water-logging for extended period. Chemical analysis is necessary to distinguish the biopolymers accumulating in the site-specific cell walls. The fact that biopolymer accumulation got restricted under water-logged condition is an interesting discovery in polyderm-bearing roots of wax apples.

When polyderm layers decreased, the amount of biopolymers accumulating in polyderm layers decreased as well. Suberin-like accumulation in the endodermis-like cell layers and lignin-like accumulation in the thick-walled cell layers existed alternatively beyond the original endodermis, comprising the polyderm. It is not known why formation of these layers got restricted in water-logged roots. What mechanism could control the new cell layer formation at the polyderm tissue? In feijoa roots, also belonging to Myrtaceae family, we reconfirmed that schematized development takes place due to cell division at the pericycle resulting in the formation of three new cell layers at a time (Nii et al., 2012). Though similar cell division pattern occurred in wax apple roots (unpublished data), cell division at pericycle stopped earlier in water-logged batches. It is thought that the metabolic energy required to form new layers and synthesize biopolymers got affected by shortage of oxygen in the soil. Further studies including measurement of ions in the soil is required to gain a better knowledge on factors affecting cell division at polyderm. On the other hand, lignin is a polymer of phenol propane units that accumulates within the cellulose strands and toughens the cell wall (Cutter, 1970). It's likely that in water-logged soil, there is less need for cells to accumulate lignin for mechanical support unlike dry soil. The multiple layers of endodermis-like cells existing beyond the original endodermis could be functioning like multiple filters but there is no

need for additional endodermis-like cell layers in water-logged roots. It can be speculated that polyderm layers may increase under drought stress conditions.

In young roots, fewer cortical cells had lignin-like cell wall encrustation in water-logged batches. This result is similar to data by Song et al. (2011) showing that crescent-like lignin thickenings appeared less in water-logged red bayberry root cortex than in normal and dry batches. Not all cortical cells accumulated lignin and only certain areas of the cell wall accumulated lignin resembling the letter “u”, closed centripetally. It is known that plant cells can modify their cell walls in many ways with subcellular precision but much remains unknown about mechanism that controls such site-specific cell wall modification at molecular level (Roppolo and Geldner, 2012).

Lastly, aerenchyma formed vigorously in young roots of water-logged roots than ones grown normally. Aerenchyma formation is known to reduce flood stress by allowing an internal pathway for oxygen to root zone to aid in respiration and oxidation (Armstrong et al., 1994; Schussler and Longstreth, 2000). A significant amount of research concerning aerenchyma has been conducted in cereal crops like *Zea Mays* (Campbell and Drews, 1983) and *Oryza Sativa* (Webb and Jackson, 1986) and more recently in the grass, *Spartina alterniflora* (Maricle and Lee, 2002). Such herbaceous plants have short lifespan compared to perennial trees that possess a persistent root system. Therefore, fruit trees could form more persistent aerenchyma so developmental regulation of aerenchyma formation in woody fruit tree roots is necessary at histological level. In this study, young and older roots were examined. It is evident from this study, that aerenchyma existed in cortical area as long as the cortex was intact but ceased to exist when the cortex sloughed off. Shedding of primary root tissues like epidermis, cortex and endodermis seem to be a natural process in healthy perennial plants and has

been reported since early times by Head (1968).

Though anatomical study alone is not enough to gain a deeper understanding of structural changes occurring in roots grown in water-logged conditions, it is clear from this study that root polyderm layers decreased under water-logged treatment and this may be a newly uncovered result as far as Myrtaceae fruit trees are concerned. Further experiments on root response to varying degrees and periods of flooding should be carried out to broaden our understanding on the role of structural changes in root tissues and its possible effect on productivity of wax apple trees in the long run.

## **2.5 Summary**

Tropical regions are prone to seasonal rains that leave the soil water-logged for long periods. Studying the impact of long-term water-logging on root development is important for fruit tree cultivation in the tropics. Myrtaceae roots are known to develop a protective tissue called polyderm. Among many Myrtaceae fruit trees, we observed that wax apple roots bear the most number of polyderm layers. How do polyderm-bearing roots respond to water-logged condition? In this anatomical study, young and older root tissues of three-year-old wax apple trees grown in normal and long-term water-logged conditions were compared. Root specimens sectioned by freehand or with an ultramicrotome were stained and observed under a fluorescent or optical microscope. Under normal condition, up to 5 layers of polyderm layers, composed of alternating endodermis-like cell layers and thick-walled cell layers with lignin and suberin accumulation, were observed, whereas in water-logged condition, polyderm layers got restricted to 2-3 layers. In young water-logged roots, lignin-like accumulation in the inner cortical cell wall was fewer than in normal roots. Aerenchyma

developed dramatically in cortical area of roots grown in water-logged condition signaling the hypoxic nature of the soil environment. Decrease of polyderm layers in water-logged condition indicates the insignificance of adding new cell layers with modified cell walls beyond the endodermis. Further experiments must be carried out in varying degrees and periods of water-logging to understand the mechanism controlling cell wall modification in root tissues and to clarify the role of structural changes and its possible long run effects.

## 2.6 References

- Armstrong, W., R. Brandle, M.B. Jackson. 1994. Mechanism of flood tolerance in plants  
*Acta. Bot. Neerl.* 43: 307-358.
- Brundrett, M.C., D.E. Enstone, C.A. Peterson. 1988. A berberine-aniline blue  
fluorescent  
staining procedure for suberin, lignin and callose in plant tissue. *Protoplasma* 146:  
133-142.
- Campbell, R., M.C. Drew. 1983. Electron microscopy of gas space (aerenchyma)  
formation in adventitious roots *Zea mays* L. subjected to oxygen shortage. *Planta* 157:  
350-357.
- Cutter, E.G. 1970. *Plant Anatomy: Experiment and Interpretation*. Edward Arnold,  
London p. 39.
- Esau, K. 1970. *Anatomy of Seed Plants*. 2nd Edition. John Wiley and Sons (New York)  
p.185.
- Fahn, A. 1982. *Plant anatomy*. 3rd Edition. Pergamon Press (Oxford) p.368.
- Head, G.C. 1968. Seasonal changes in the diameter of secondary thickened roots of fruit  
trees in relation to growth of other parts of the tree. *J. Hort. Sci.* 43: 275-282.
- Hsu, Y.M., Tseng, M.J., Lin, C.H. 1999. The fluctuation of carbohydrates and nitrogen  
compounds in flooded wax-apples trees. *Bot. Bull. Acad. Sin.* 40: 193-198.
- Jensen, W.A. 1962. *Botanical Histochemistry - principles and practice*. University of  
California, Berkeley, W.H. Freeman and Company p. 205
- Komatsu, S., Kobayashi, Y., Nishizawa, K., Nanjo, Y., Furukawa, K. 2010.  
Comparative  
proteomics analysis of differentially expressed proteins in soybean cell wall during

flooding stress. *Amino Acids* 39: 1435-1449.

Kozlowski, T. 1984. *Flood & Plant Growth*. Academic Press (Orlando, Florida) pp. 129-163.

Lux, A., Morita, S., Abe, J., Ito, K. 2005. An improved method for clearing and staining free-hand sections and whole-mount samples. *Ann. Bot.* 96: 986-996.

Maricle, B.R., Lee, R.W. 2002. Aerenchyma development and oxygen transport in the estuarine cordgrasses *Spartina alterniflora* and *S. anglica*. *Aquat Bot.* 74: 109-120.

Morton, J. 1987. Java Apple. In: *Fruits of Warm Climates*. Julia F. Morton (Miami) pp. 381-382.

Nii, N., Ohtsuka, S., Ye, L., Song, Y. 2012. Formation of endodermis-like cells with Casparian strip and thick wall cells derived from pericycle in the roots of *Feijoa sellowiana* (Myrtaceae). *J. Japan. Soc. Hort. Sci.* 81: 314-319.

Nii, N., Pan, C.X., Ogawa, Y., Cui, S.M. 2004. Anatomical features of the cell wall in-growth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. *J. Japan. Soc. Hort. Sci.* 73: 411-414.

Pan, C.X., Nakao, Y., Nii, N. 2006. Anatomical development of phi thickening and the Casparian strip in loquat roots. *J. Japan. Soc. Hort. Sci.* 75: 445-449.

Roppolo, D., Geldner, N. 2012. Membrane and walls: who is master, who is servant? *Curr. Opin. Plant. Biol.* 15: 608-617.

Rowe, R.N., Beardsell, D.V. 1973. Waterlogging of fruit trees. *Hort. Abst.* 43: 534-544.

Salvador, V.H., Lima, R.B., Dos Santos, W.D., Soares, A.R., Bohm, P.A., Marchiosi, R., Maria, L.F., Osvaldo, F.F. 2013. Cinnamic acid increases lignin production and inhibits

soybean root growth. PLoS ONE 8: e69105 10.1371/ journal.pone.0069105.

Schaffer, B., Anderson, P.C., Ploetz, R.C. 1992. Responses of fruit trees to flooding. Hort.Rev. 13: 257-313.

Schussler, E. E., Longstreth, D.J. 2000. Changes in cell structure during the formation of root aerenchyma in *Sagittaria lancifolia* (Alismataceae). Amer. J. Bot. 87: 12-19.

Shu, Z., Lin, T., Lai, J., Huang, C., Wang, C., Pan, H. 2007. The industry and progress review on the cultivation and physiology of wax apple- with special reference to 'Pink' variety. Asian & Australasian J. Plant Sci. Biotech. 1: 48-53.

Song, Y., Ye, L., Nii, N. 2011. Effects of soil water availability on development of suberin

lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Horti. 129: 554-560.

Webb, J., Jackson, M.B. 1986. A transmission and cryo-scanning electron microscopy study of the formation of aerenchyma (cortical gas-filled space) in adventitious roots of rice (*Oryza sativa*). J. Exp. Bot. 37: 832-841.

Table 2-1. The development of polyderm layers in wax apple roots under normal and water-logged conditions

| Treatment<br>(Number of roots) | Degree of polyderm layers |         |         |           |
|--------------------------------|---------------------------|---------|---------|-----------|
|                                | + + <sup>z</sup>          | + + +   | + + + + | + + + + + |
| Water-logged (22)              | 8 (36) <sup>y</sup>       | 14 (64) | 0       | 0         |
| Normal (20)                    | 0                         | 8 (40)  | 8 (40)  | 4 (20)    |

<sup>z</sup> ++, +++, +++++, ++++++: Polyderm appeared in two, three, four and five layers, respectively.

<sup>y</sup> Number of roots that formed polyderm layers (percentage).

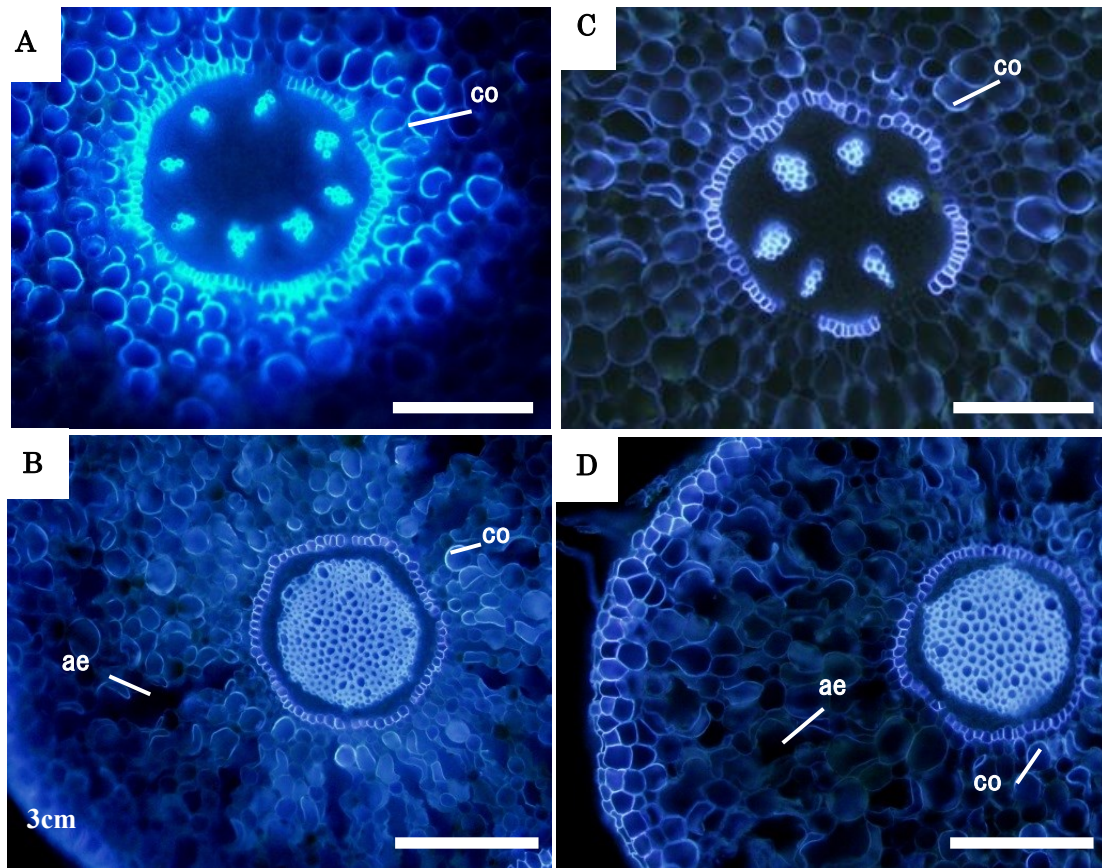


Fig.2-1. Crescent-shaped lignin accumulation in cortical cell walls of wax apple roots grown under normal and water-logged treatments. Unstained cross sections were examined under fluorescent microscopy. Panels A, B represent normal and panels C, D represent water-logged roots. Distance from the root tip is panels A, C: 10 mm; panels B, D: 30 mm; co: cortex cell with lignin accumulation; ae: aerenchyma. Scale bars = 100  $\mu$ m.

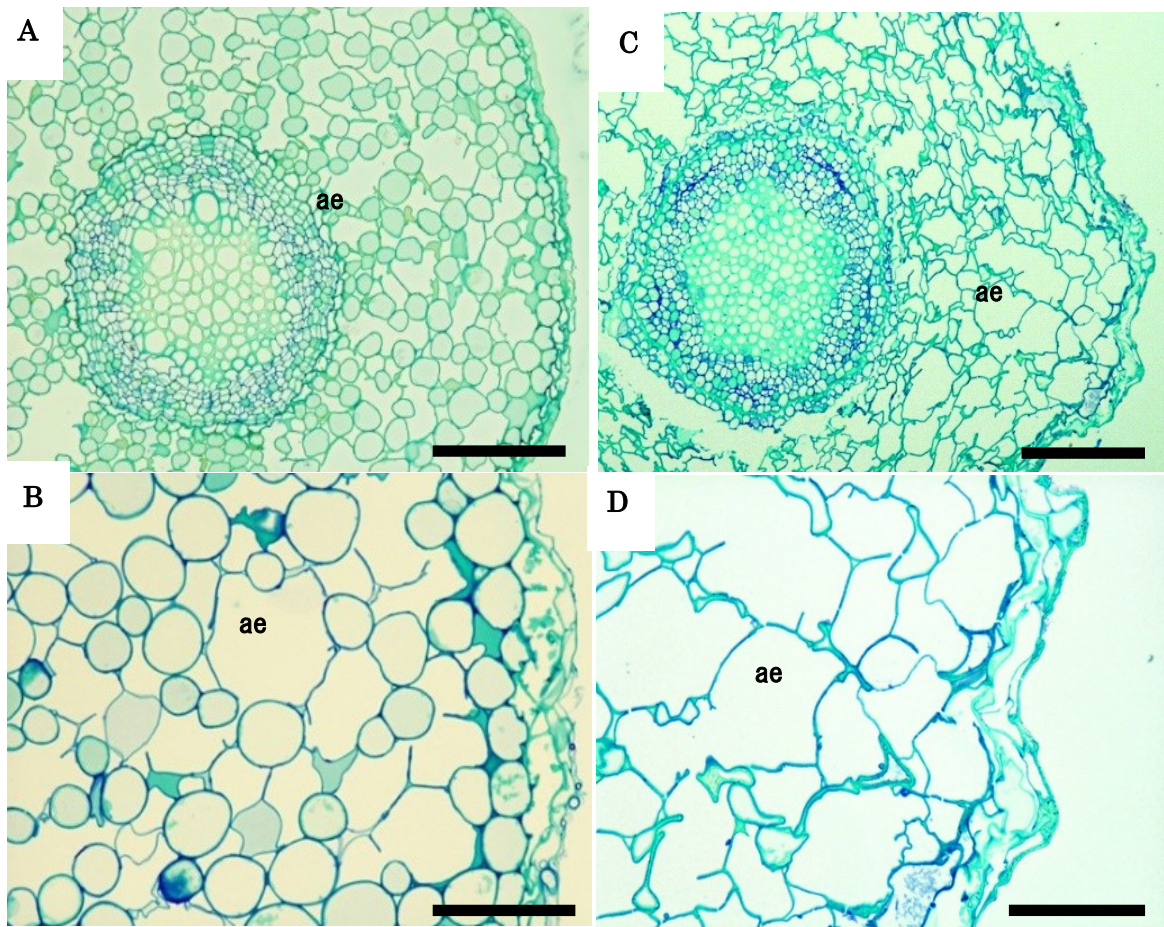


Fig.2-2. Aerenchyma formation in wax apple root grown under normal and water-logged treatments. Cross sections of root samples embedded in technovit resin were stained with methylene blue. Panels A, B represent the normally grown roots and panels C, D represent roots grown in water-logged condition. Panels B, D are enlargements of panels A, C, respectively. Distance from the root tip is panels A: 110 mm, C: 130 mm; ae: aerenchyma. Scale bars = 100 $\mu$ m (Panels B, D); 200  $\mu$ m (Panels A, C).

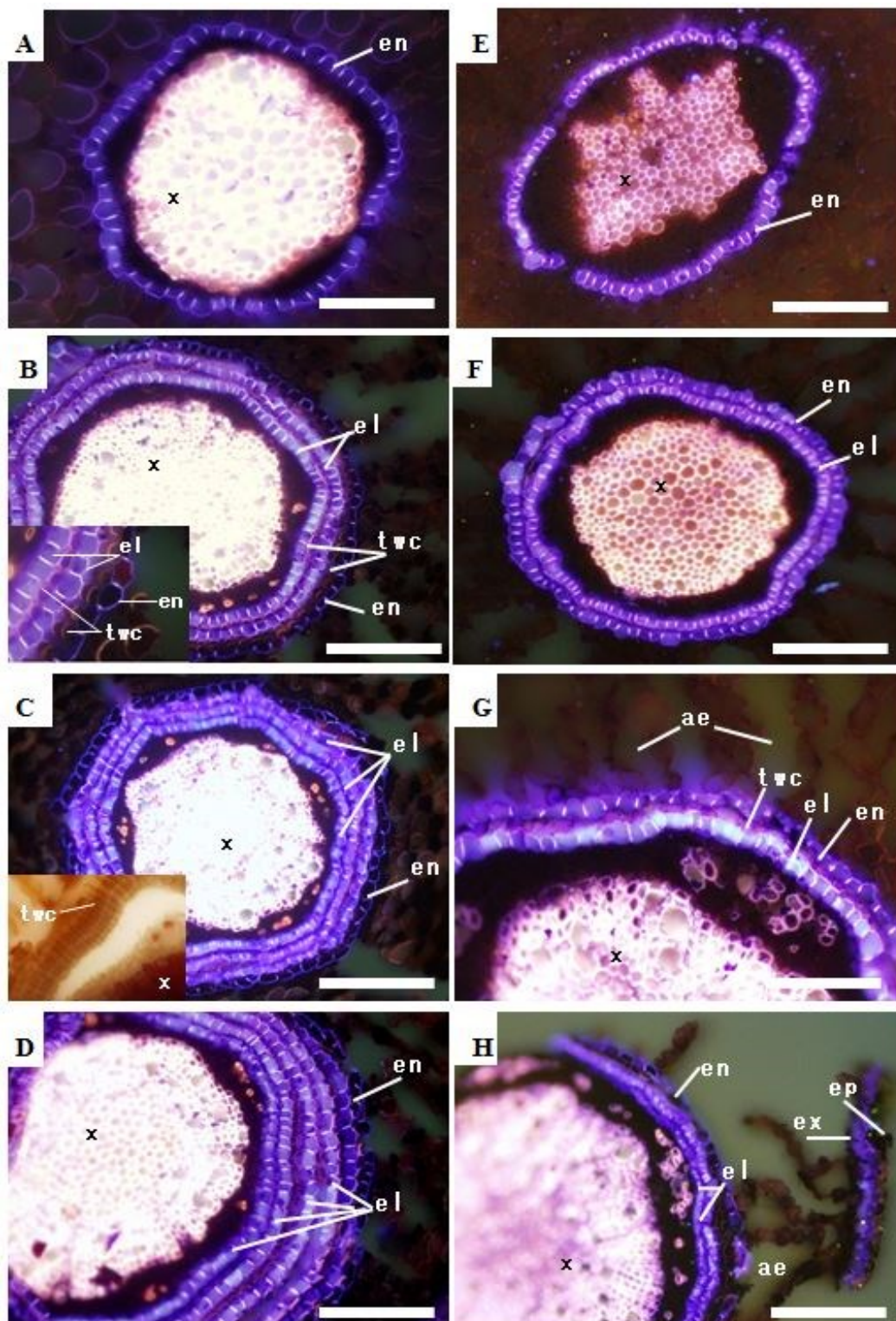


Fig.2-3. Polyderm development in wax apple roots grown under normal and water-logged treatment. Cross sections were examined under a fluorescent microscope

after staining with berberine-aniline blue-safranin O. Panels A-D represent normally grown roots and panels E-H represent roots grown in water-logged condition. In panel B, enlarged section of panel B is presented in left corner. In panel C, cross-section of polyderm layers that turned red when stained with phloroglucinol is presented in left corner. Distance from the root tip is panels A, E: 70 mm; panels B, F: 100 mm; panels C, G: 110 mm; panels D, H: 120 mm; ep: epidermis; ex: exodermis; en: endodermis; el: endodermis-like cell layer; twc: thick-walled cell layer, ae: aerenchyma, x: xylem. Scale bars = 100  $\mu$ m.

### **Chapter 3**

Effect of soil water conditions on biopolymer accumulation in root polyderm of *Feijoa sellowiana* (Myrtaceae)

### 3.1 Introduction

*Feijoa sellowiana*, a subtropical species of Myrtaceae family, native to South Brazil with secondary dispersion in Uruguay (Thorp and Bieleski, 2002), has both ornamental and medicinal properties and shows great potential in foods, drugs and cosmetics industry (Hardy and Michael, 1970, Kolesnik et al., 1991). Biopolymers like lignin and suberin are cell wall components and specialized plant cells can locally modify their cell walls by accumulating biopolymers as part of a developmental program or as a response to environmentally challenging conditions (Roppolo and Geldner, 2012). *Feijoa* is an interesting species to study biopolymer accumulation because its root bears a special type of protective tissue called polyderm composed of alternating suberized and non-suberized layers, common in only a few other plant families like Hypericaceae, Onagraceae and Rosaceae (Esau, 1970; Fahn, 1982). Our previous studies on non-polyderm bearing roots of loquat (*Eriobotrya japonica* Lindl.) and red bayberry (*Myrica rubra* Sieb. et Zucc.) showed that biopolymer accumulation in cortical cell walls increased in drought conditions. *Phi*-thickening, a kind of cell wall ingrowth that gets impregnated with lignin developed markedly in loquat root cortex (Nii et al., 2004; Pan et al., 2006) and crescent- shaped lignin thickenings increased in red bayberry cortex under drought stress (Song et al., 2011). However, studies on biopolymer accumulation in polyderm-bearing roots are rare. Recently, we examined the effect of long-term water-logged condition in another species of Myrtaceae, wax apple (*Syzygium samarangense*) and found that polyderm layers decreased in water-logged roots than normally grown roots (unpublished data). To understand what restricted cell division at polyderm, a more detailed study on biopolymer accumulation is necessary. In this study we examined whether biopolymer accumulation in polyderm

was affected by varying the soil water condition in roots of feijoa.

### **3.2 Materials and Methods**

In years 2011 and 2012, 2-3 year-old feijoa (cultivar 'Mammoth') trees, propagated from cuttings, were grown under water-logged, normal and drought conditions in a greenhouse located at Meijo University in Nagoya, Japan. They were grown in plastic baskets (diameter and depth 300 mm, stitch size 30 mm) covered with nonwoven fabric and filled with sandy soil. Eight pots were allotted per treatment batch in both years. Treatment was maintained for more than 4 months starting from early April to late August of each year.

The water-logged pots were watered every day until it saturated the soil in the pots, and leaked out of pot holes and collected in plastic dishes placed at the bottom of the pots. Water depth in these dishes was maintained at a depth of several centimeters. Pots treated normally were watered only when the upper surface of the soil became dry. No dish was placed under pots in normal and drought treatments. Depending on the weather, it took 2-4 days for the upper surface to dry. The magnitude of stress during the treatment was assessed by measuring the water potential using a pressure chamber (Plant Moisture Stress Measurements by PMS Instrument Company, Oregon, U.S.A) in the second year as shown in Fig. 2. A leaf attached to petiole on second or third node was collected from each plant in air-tight plastic bag and delivered to the lab in an icebox for immediate measurement of water potential before noon. The value of the pressure chamber was maintained at approximately  $-1.0$  MPa for water-logged,  $-2.0$  MPa for normal and  $-3.0$  MPa for dry treatment before watering the pots. To collect roots, the plants were gently removed from their containers without disturbing all the

soil that was intact with the root mass. New white roots were collected from the tip to the basal zone. Then they were hand-sectioned using a stainless steel razor blade immediately after collection. Serial transverse sections were prepared at less than 20 mm from the root tip, between 30-50 mm from the root tip and above 60 mm from the root tip to examine changes in structural development as the root aged. Root specimens were then examined under fluorescent microscopy (excitation wavelength 365nm) without staining. About 20-30 roots were sectioned and observed in each treatment. Then a representative root was selected for report since the basic anatomical features were similar among root samples. Firstly, the distance from the root tip when thick-walled cells developed between the endodermis and the endodermis-like cells, was examined. Secondly, the extent of biopolymer accumulation in thick-walled cell layers was compared as—: thick-walled cells developed but biopolymer accumulation was not observed, +—: biopolymer accumulation was observed in a few thick-walled cells, +: light biopolymer accumulation was observed in all thick-walled cells, ++: heavy biopolymer accumulation was observed in all thick-walled cells as shown in Fig. 1.

### **3.3 Results and Discussion**

Feijoa root is interesting for the study of biopolymer accumulation because it bears polyderm, a protective tissue comprising alternating cell layers with suberized and non-suberized cell layers. In our previous study (Nii et al., 2012)., we called the suberized layers, endodermis-like cell layer (el) because it contained the Casparian strip and the non-suberized layers, thick-walled cell (twc) layers because biopolymer accumulation thickened the cell wall of these layers. When stained with

phloroglucinol-HCl, it turned red so it was thought to contain mostly lignin. The objective of this study was to confirm whether lignin-like biopolymer accumulation in twc, got affected under different soil water conditions. Based on our comparative observation of roots at the same distance from the root tip and with similar xylem vessel amount, we concluded that biopolymer accumulation in the thick-walled cells was earlier in roots grown in drought treatment (Figs. 3C, 1) and later in water-logged roots (Figs. 3B, B1) compared to normally grown roots (Figs. A, A1) in both years. Biopolymer accumulation increased with distance from the root tip in every treatment (Figs. 4, 5). Lignin-like accumulation was ++ level in all roots in the drought treatment whereas in water-logged roots, biopolymer accumulation was – level in as seen in Figs. 2B, B1.

In roots under drought treatment, biopolymer accumulated earlier and more heavily and this result was similar to roots of loquat (Nii et al., 2004; Pan et al., 2006) and red bayberry (Song et al., 2011). Further study using chemical analysis is necessary to distinguish whether biopolymer accumulating in thick-walled cells is solely lignin. Recently biopolymer in root tissues gained attention because lignin was detected in the Casparian strip of arabidopsis (Naseer et al., 2012). Detailed knowledge on biopolymer accumulation may hold the key to understanding the function of polyderm tissue. In this study, our focus was mainly on the extent of biopolymer accumulation in thick-walled cells in different soil water conditions. However, we also noticed that many thick-walled cell layers existed one after another, between the endodermis and endodermis-like cell layer in roots under drought treatment (Fig.3.C1) than normal treatment (Fig.3. A). Cell layers existing alternately in the order of “suberized - non-suberized - suberized” was disturbed under drought treatment. Further studies must

be conducted to elucidate how thick-walled cell layers increase in roots of drought treatment because it differed from our previous conclusion that each cell in the pericycle divided into 3 cell layers; the outermost cell layer accumulated lignin to form two, the second layer accumulated suberin to form the el and the innermost cell layer retained the pericyclic characteristic and divided further in the same way to form the polyderm layers (Nii et al., 2012). Irregularity in biopolymer accumulation at polyderm layers under drought condition must be examined further.

### **3.4 Summary**

Feijoa root bears a special type of protective tissue called polyderm composed of alternating suberized and non-suberized layers. Recently, we examined the effect of long-term water-logged condition in another species of Myrtaceae, wax apple (*Syzygium samarangense*) and found that polyderm layers decreased in water-logged roots than normally grown roots (Tuladhar et al., 2015). To understand what restricted cell division at polyderm, a more detailed study on biopolymer accumulation is necessary. In this study we examined whether biopolymer accumulation in polyderm of feijoa root was affected by varying the soil water condition.

2-3 year-old feijoa (cultivar ‘Mammoth’) trees, propagated from cuttings, were grown under water-logged, normal and drought conditions. Eight pots were allotted per treatment batch. The magnitude of stress during the treatment was assessed by measuring the water potential using a pressure chamber. The value of the pressure chamber was maintained at approximately  $-1.0$  MPa for water-logged,  $-2.0$  MPa for normal and  $-3.0$  MPa for dry treatment before watering the pots. Root specimens were then examined under fluorescent microscopy (excitation wavelength 365nm) without

staining. About 20-30 roots were sectioned and observed in each treatment.

The objective of this study was to confirm whether lignin-like biopolymer accumulation in twc, got affected under different soil water conditions. Based on our observation, we concluded that biopolymer accumulation in the thick-walled cells was earlier in roots grown in drought treatment and later in water-logged roots compared to normally grown roots. Biopolymer accumulation increased with distance from the root tip in every treatment. In roots under drought treatment, biopolymer accumulated earlier and more heavily. We also noticed that cell layers existing alternately in the order of “suberized-non-suberized-suberized” was disturbed under drought treatment. Irregularity in biopolymer accumulation at polyderm layers under drought condition must be examined further.

### **3.5 References**

- Esau, K. 1970. Anatomy of Seed Plants. 2nd Edition. John Wiley and Sons (New York) p.185.
- Fahn, A. 1982. Plant anatomy. 3rd Edition. Pergamon Press (Oxford) p.368.
- Hardy, P.J and B.J. Michael, 1970. Volatile components of feijoa fruits. Phytochem. 9: 1355-1357.
- Kolesnik,,A.A., Golubev, V.N., Gadzhieva, A.A.1991. Lipids of the fruit of Feijoa sellowiana. Chem. Nat. Comp. 27: 404-407.
- Naseer S, Lee Y, Lapierre C, Franke R, Nawrath C, Geldner N 2012. Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. Proc. Natl. Acad. Sci. USA 109: 10101-10106.
- Nii, N., S. Ohtsuka, L. Ye, Song Y. 2012. Formation of endodermis-like cells with

Casparian strip and thick wall cells derived from pericycle in the roots of *Feijoa sellowiana* (Myrtaceae). J. Japan. Soc. Hort. Sci. 81: 314-319.

Nii, N., Pan C. X., Ogawa, Y., Cui, S. M. 2004. Anatomical features of the cell ingrowth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. J. Japan. Soc. Hort. Sci. 73: 411-414.

Pan, C. X., Nakao, Y., Nii, N. 2006. Anatomical development of *phi* thickening and the Casparian strip in loquat roots. J. Japan. Soc. Hort. Sci. 75: 445–449.

Roppolo, D., Geldner, N. 2012. Membrane and walls: who is master, who is servant? Curr. Opin. Plant Biol. 15: 380-383.

Thorp, G., Bielecki, R. 2002. Feijoas: Origins, Cultivation and Uses. 1<sup>st</sup> Edition. David Bateman Ltd, p: 7

Song, Y., Ye, L., N. Nii. 2011. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Horti. 129: 554-560.

Tuladhar, A., Ohtsuka, S., Nii, N. 2015. Anatomical study on wax apple (*Syzygium samarangense*) roots under long-term water-logged conditions. Trop. Agr. Develop. 59: 1-6.

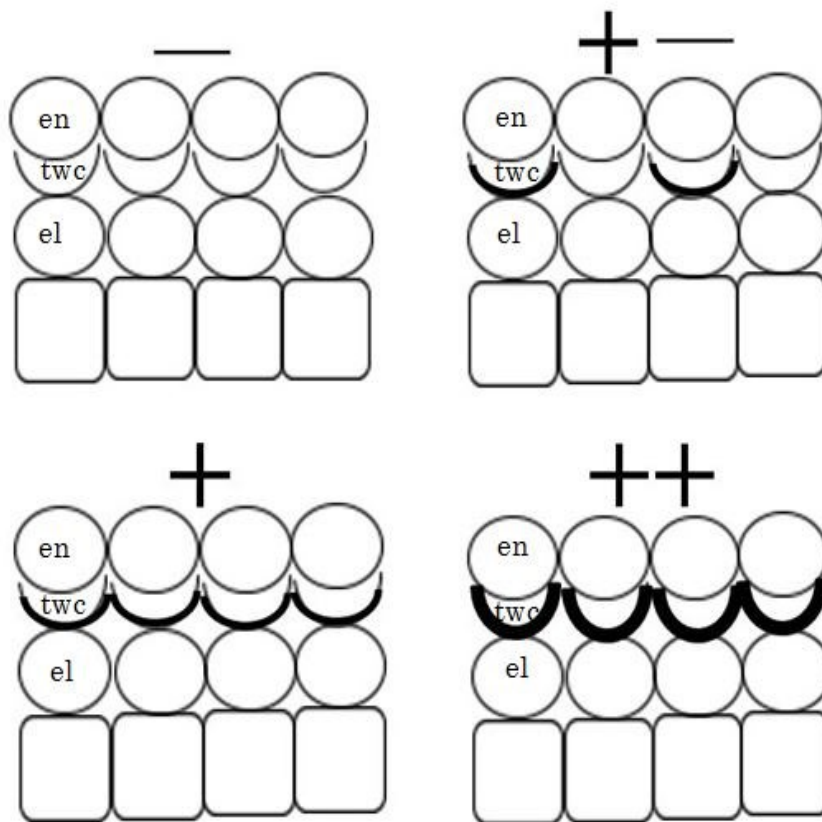


Fig.3-1 Illustration on the extent of lignin accumulation in thick-walled cells at polyderm. en: endodermis, twc: thick-walled cell, el: endodermis-like cell.

- thick-walled cells developed but lignin accumulation was not observed.
- + — lignin accumulation was observed in some thick-walled cells.
- + lignin accumulation was observed in all thick-walled cells.
- + + heavy lignin accumulation was observed clearly in all thick-walled cells.

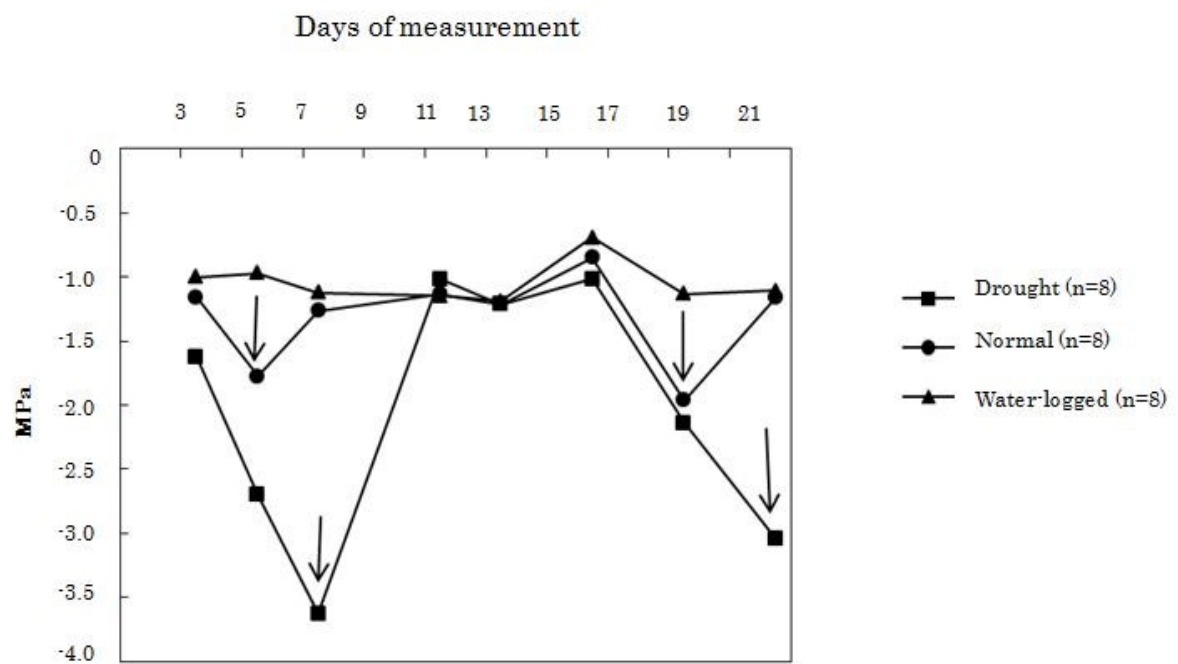


Fig.3-2 Water-potential measurement of feijoa roots grown in drought, normal and water-logged treatments in the year 2012.

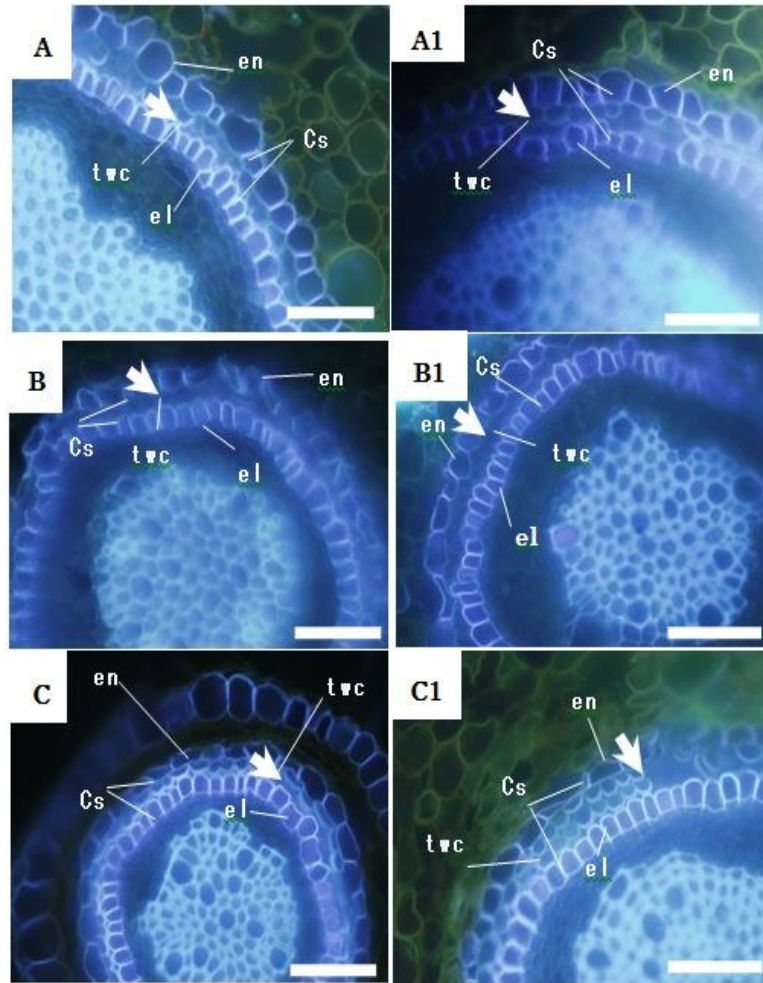


Fig.3-3. Difference in biopolymer accumulation in thick-walled cells (twc) in roots grown in normal (Panels A, A1), water-logged (Panels B, B1), drought (Panels C, C1) treatments in the consecutive years 2011 (Panels A, B, C) and 2012 (Panels A1, B1, C1). Arrowhead shows the extent of biopolymer accumulation in thick-walled cells. en: endodermis, Cs: Casparian strip, twc: thick-walled cells, el: endodermis-like cells. Distance from the root tip for all panels 120 mm. Scale bar = 50  $\mu$ m.

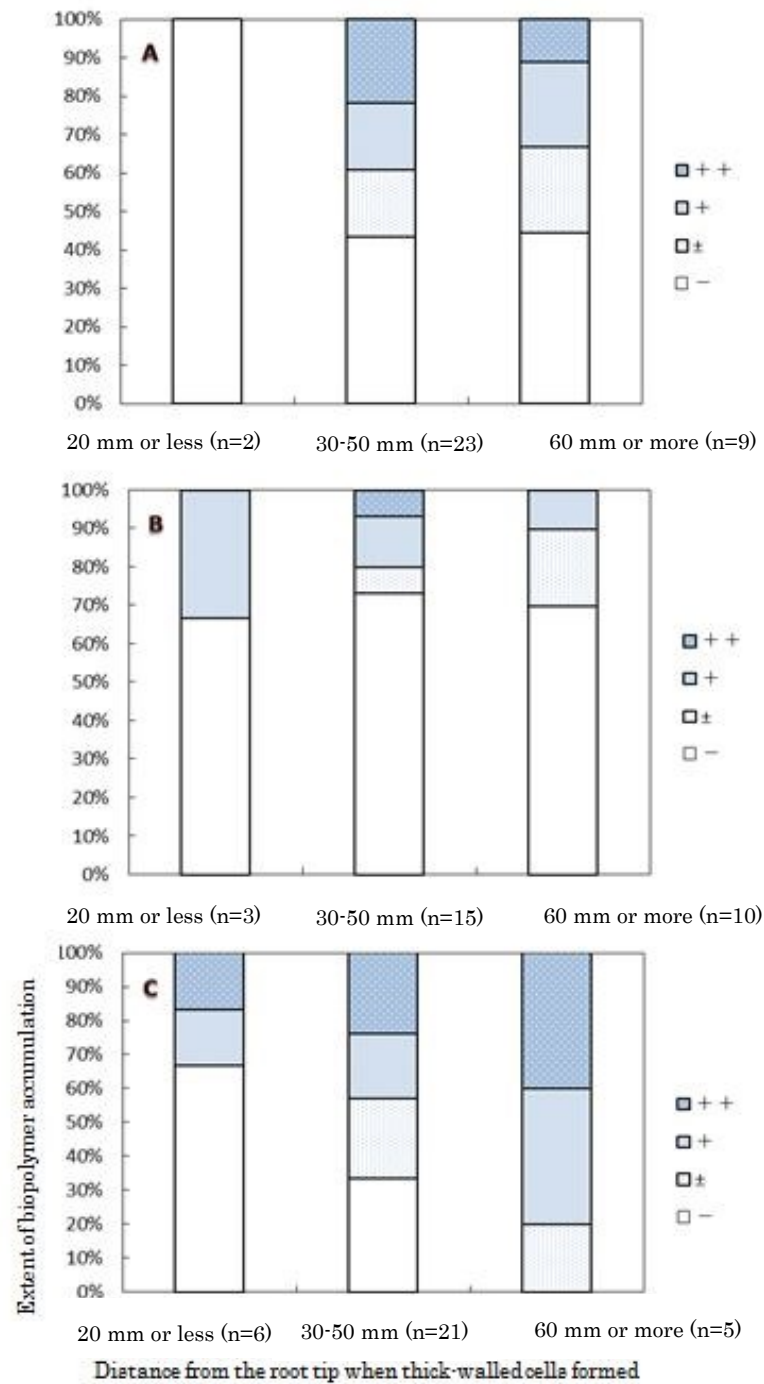


Fig.3-4 Biopolymer accumulation according to distance from the root tip in the year 2011.

A: normal, B: water logged, C: drought

-, +, ++: Extent of biopolymer accumulation in thick-walled cells.

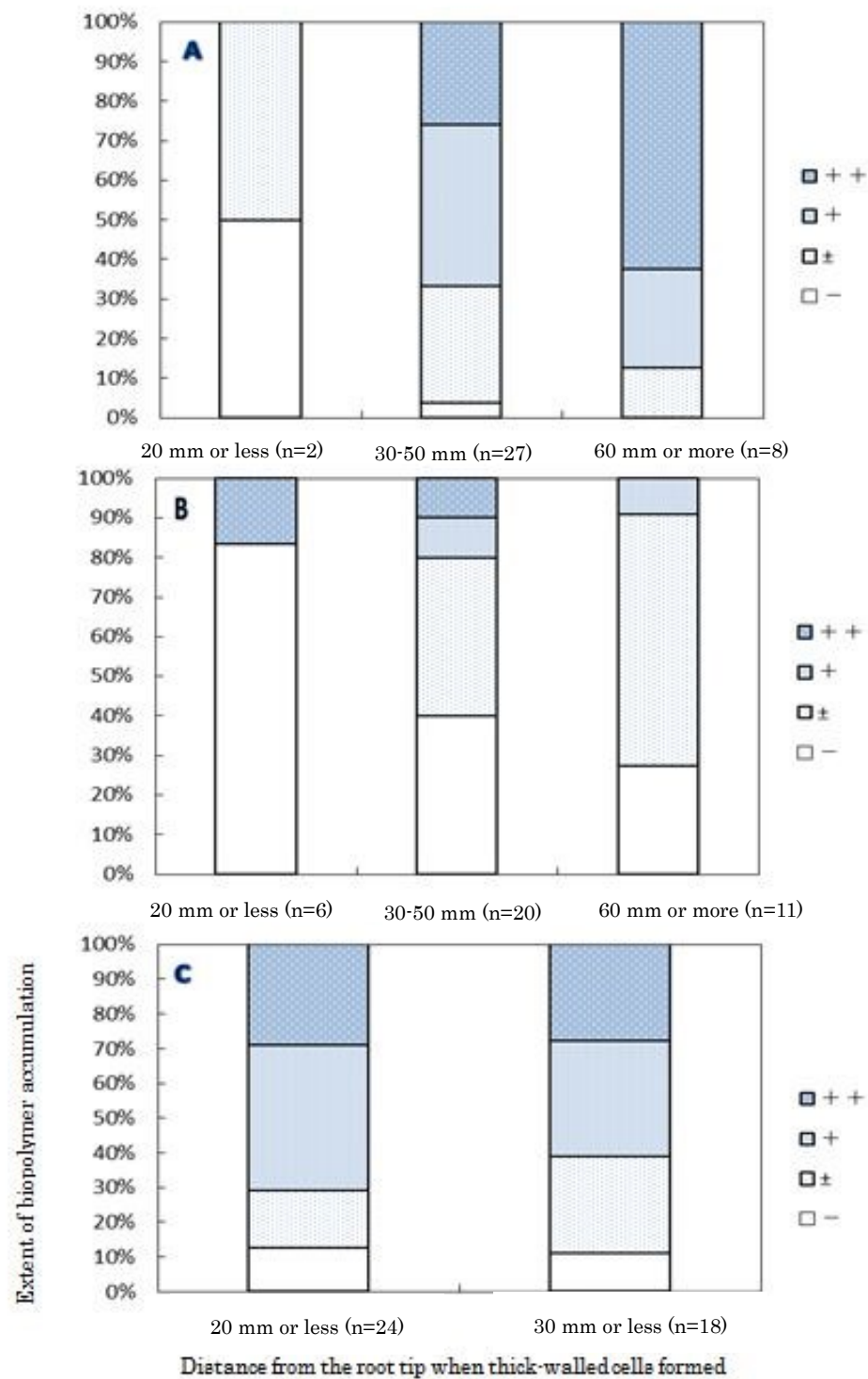


Fig.3-5 Biopolymer accumulation according to distance from the root tip in the year 2012.

A: normal, B: water logged, C: drought

-, +, ++: extent of biopolymer accumulation in thick-walled cells.

## **Chapter 4**

**An anatomical study of developmental changes in maturing root tissues of pomegranate (*Punica granatum* L.) and formation of a unique type of periderm**

## 4.1 Introduction

Pomegranate (*Punica granatum* L.) belongs to Punicaceae family of plants. It is an oldest known edible fruit tree species originating from central Asia which is now produced worldwide for its highly recognized medicinal, nutritional and ornamental properties (Teixeira da Silva et al., 2013). Its adaptive characteristics reflect in its geographically wide distribution. Roots are directly affected by different soil environments. Maintaining a healthy root system is crucial for a steady yearly production, especially in the case of deciduous fruit trees such as pomegranates that relies on starch deposited in roots over winter. Unfortunately, there is very limited data on pomegranate roots. A study on pomegranate root distribution was reported recently by Hiwale et al., (2011) but an anatomical study like the present study is still rare. One reason could be because pomegranates are known to have toxic bark and roots (Tripathi and Singh, 2000).

In this study, pomegranate roots were investigated anatomically to gain basic knowledge of root tissue development from growth to maturity. Recording changes that occur during root growth may further clarify tissue function and point out interesting characteristics unique to pomegranate roots. In the past, we have reported similar works in grapevine roots (Song et al. 2011b, Tuladhar and Nii, 2013) with special focus on the Casparian strip. In the present study, our focus is mainly on biopolymer accumulation during periderm formation.

Early observations on polyderm were reported by Luhan (1955) in *Unserer Alpenpflanzen* and by Nelson and Wilhelm (1957) and Mann (1930) in strawberry roots. Esau (1970) described polyderm as a protective tissue composed of alternating suberized and non-suberized cell layers. According to Esau (1970) polyderm is found in

only a few plant families such as Myrtaceae, Hypericaceae, Onagraceae and Rosaceae families but Punicaceae was not mentioned.

Recently, we studied the roots of feijoa (*Feijoa sellowiana*) belonging to Myrtaceae and reconfirmed the features of the polyderm development (Nii et al., 2012). Based on our observations, polyderm in feijoa roots formed due to the division of a single-layered pericycle into three layers at a time. Lignin accumulated in the outermost layer, immediately beyond the endodermis, and we called that layer thick-walled cell layer (twc). Suberin accumulated in the middle layer forming the Casparian strip so we called that layer endodermis-like cell layer (el) and the innermost layer retained the pericyclic characters so it divided further to form a new set of three layers. During this observation, our attention was drawn to the accumulation pattern of lingo-suberic materials. At the polyderm, biopolymers mainly lignin and suberin accumulated on opposite sides of the cell wall in two separate cell layers. Lignin-like deposit was “closed” centripetally resembling the letter “w” at the thick-walled cells whereas suberin-like deposit was “open” centripetally resembling the letter “m” at the endodermis-like cells and this accumulating pattern was commonly detected in roots of five Myrtaceae species (Tuladhar et.al, 2014).

For woody roots, accumulation of biopolymers such as lignin and suberin is of particular importance because it is known to protect plants from fungal diseases and insect attacks (Moerschbacher et al., 1900, Nelson and Wilhelm, 1957, Williamson, 1984). The accumulation of biopolymers such as lignin and suberin is a species-specific genetic feature and seems to be useful as a barometer to distinguish species. For example, *phi*-thickening is a kind of lignin accumulation found in Rosaceae species such as apples (Mackenzie, 1979) and loquats (Nii et al., 2004, Pan et al., 2006). In

addition, our data shows that unfavorable growth conditions like flooding or drought affects the accumulation of these biopolymers. Our studies revealed that under drought conditions, *phi*-thickenings developed markedly in cortex cells of loquat trees (Nii et al., 2004, Pan et al., 2006). Similarly, in cortex of red bayberry roots, crescent-shaped lignin accumulation increased under drought stress (Song et al., 2011a). Thus we felt the need to trace the biopolymer accumulation in root tissues of pomegranate roots from growth to maturity.

In this preliminary study, pomegranate roots grown in normal condition were continuously sectioned from the root tip to basal area to examine 1) the accumulation of lingo-suberic material in root tissues such as rhizodermis (ep), exodermis (ex), cortex (co), and endodermis (en) and 2) the periderm formation process during secondary growth. The results obtained from this observation are intended to be used for further stress-response related experiments. Compared to the polyderm found in Myrtaceae, in pomegranate roots, the alternating layers of suberized and non-suberized layers described as polyderm did not occur. Instead cell layers with heavy lignin-like accumulation were observed in the outermost cell layers. In the recent years, root biology is steadily gaining attention. In such a context, the present study about biopolymer accumulation in protective root tissues of woody roots during its maturing phase is highly appropriate to broaden our knowledge about its function which still needs much clarification.

## **4.2 Materials and Methods**

Approximately ten-year-old pomegranate trees (cv. ‘Wonderful’) which were propagated from cuttings and grown under normal conditions in a greenhouse at Meijo

University were used for the present investigation. The pomegranate cuttings were grown in plastic pots (13 cm wide and 11 cm deep) filled with sandy soil and watered when the upper surface of the soil became dry. The growth of pomegranate trees was dwarfed like bonsai by using small-sized plastic pots. Roots were collected from twenty different trees for observation. To collect roots, plants were removed from the pots and only roots with new white root tips were collected from the root tip to basal area.

Hand-sections of roots were prepared by using a new stainless steel razor blade. Serial transverse sections, commencing at approximately 5 mm from the root tip toward the basal portion of the root, were cut at 10 mm intervals to examine changes in structural development as roots matured.

Root specimens were examined under fluorescent microscopy (excitation wavelength 365 nm) with or without staining. Several different staining solutions, including berberine hemisulfate-aniline blue-safranin O were used (Brundrett et al., 1988). Lux et al., (2005) reported a novel method for simultaneously clearing and staining tissues in which the fluorochrome 0.1% berberine hemisulfate was dissolved in a clearing mixture consisting of pure lactic acid saturated with chloral hydrate. We used this method and incubated the root sections in 0.1% berberine hemisulfate solution at 40°C for 30 minutes. The sections were then counterstained with 0.5% aniline blue (dissolved in H<sub>2</sub>O) at room temperature for 30 minutes followed by safranin O for 1 minute. All specimens were then placed on a drop of H<sub>2</sub>O on a slide glass and observed under ultraviolet light using fluorescent microscopy.

Parts of same root samples used for hand sectioning were fixed in 3 % glutaraldehyde (TAAB Laboratories, England, UK), in 0.1 M cacodylate buffer, (Sigma Aldrich, Japan, pH 7.2) and stored at 4°C to examine the anatomical features of cell

development. The samples were then dehydrated through graded ethanol series and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany). Semi-ultra thin sections (1.5  $\mu\text{m}$ ) were cut using a glass knife then stained with methylene blue for histological examination under a BX 60 optical microscope (Olympus, Tokyo, Japan).

Root age was determined by surface color (i.e., white roots are young and brown roots are old) and by the distance from the root tip. Although more than twenty roots were sectioned and observed, a representative root was selected for report since the basic anatomical features were similar among root samples.

### 4.3 Results

#### *Accumulation of lingo-suberic material in pomegranate root tissues*

A scanned view of representative main root is presented as Fig. 1. The markings on it are the areas where root specimens were collected for observation on the main root. It is clear that the new root is white up to Fig.1 D and as it matures, it gradually turns brown beyond this point. The developmental changes in root tissues were examined one by one. The rhizodermis appeared to be reddish-orange at a distance of 10 mm from the root tip, implying lignin-like accumulation in its cell wall (Fig. 2A). At a distance of 20 mm from the root tip, the exodermis appeared to be milky white color implying suberin-like phenolic substance deposit in its cell wall (Fig. 2B). At a greater distance from the root tip, a clearer Casparian strip was visible in the exodermis (Figs. 2B-F). It is clear from this observation that in young, new roots, the exodermal Casparian strip forms in one or more layers of the exodermis as roots mature (Figs. 2E, F). In Fig. 3, the developmental changes in the endodermis are presented. These developmental changes in the

endodermis could be divided into successive stages I, II, III as Lux and Luxova (2001) and Maxkenzie (1979) have done to study endodermis. Endodermal cells of vascular plants may develop in three developmental states resulting in primary, secondary or tertiary endodermis, respectively (von Guttenberg, 1968). At a distance of 20 mm from the root tip, a small line-like Casparian strip appeared at the endodermis initially (Fig. 3 A). This stage was termed stage I since differentiation of the Casparian strip occurred. At a greater distance from the root tip, suberin accumulated in the entire cell wall at the endodermis (Figs. 3B, C). This stage was termed stage II since all wall areas got suberized. Eventually, the Casparian strip was observed at both the exodermis and the endodermis (Figs. 2B, 3B). At the exodermis, Casparian strip developed at a distance of 20 mm from the root tip (Fig. 2B) whereas at the endodermis, it developed at 60-80 mm from the root tip (Figs. 3B, C). It was clear that the Casparian strip developed earlier at the exodermis than the endodermis. From the distance of about 80 mm (Fig. 3C), some cells in the cortex surrounding the endodermis accumulated lignin in the cell wall. Such cortical cells accumulating lignin increased at a greater distance from the root tip (Figs. 2F, 3F). This kind of lignin accumulation was similar to crescent-thickening in cortical areas of red bayberry roots (Song et al., 2011a).

#### *Formation of lignified cell layers during secondary growth*

The development of new cells with lignin accumulation is presented in Figs. 3D-F and Fig. 4. When the surface color of roots changed from white to brown, periderm layers began to develop beyond the endodermis. At first, lignin-like deposit was observed in the endodermis cells with Casparian strip (Figs. 3D, E and 4A, B). This stage of endodermal development was termed as stage III because endodermal cell walls thickened with added

layers of lignin. Then new cell layers formed due to cell division at the pericycle (Figs. 5 A, B). Then as the central stele grew rapidly and xylem vessels increased, up to 3-4 new cell layers with lignin accumulation developed due to cell division at the phellogen (Figs. 4 C, D, Figs. 5 C, D) and all these new cell layers appeared milky white on the periphery and reddish orange on one side, “closed” centripetally. It implies that both biopolymers accumulated in the same cell layer. If tissues that stain reddish orange (mainly lignin) are scrutinized, difference in accumulation area was noticed. At the rhizodermis lignin-like accumulation appeared to be “open” centripetally resembling the letter “m” (Fig. 2) whereas it appeared to be “closed” centripetally resembling the letter “w” at the cortical cells (Figs. 2F, 3F, 4A, B), the endodermis (Figs. 3 E, F, 4A, B) and the new cells forming beyond the endodermis (Figs. 4C, D).

In Fig.6, developmental stages of these new cell layers occurring beyond the endodermis are presented. After the outermost tissues such as rhizodermis, exodermis, cortex, endodermis sloughed off due to the continuous enlargement of the central stele, new cell layers increased layer by layer to form up to 5-10 stratified layers (Figs. 6B, C). Each cell elongated tangentially and appeared to be rectangular or square-shaped (Figs. 6E, F). As roots matured, lignin-like deposit seen as vibrant reddish-orange auto fluorescence, became darker and broader in each cell (Fig. 6F). This kind of brick-like layout was quite unfamiliar to the traditional description of polyderm with alternating layers of suberized and non-suberized cell layers beyond the endodermis. In pomegranate roots, cell division at the phellogen was the cause of new cell layer formation in roots undergoing secondary growth (Figs. 5 C, D) and accumulation of what seems to be mainly lignin and suberin occurred in the same cell layer. Therefore we concluded that it may be a unique type of periderm specific to Punicacea family.

As the root aging progressed, the central stele continued to widen in diameter causing the outermost layers shed off unevenly leaving triangular chunks of lignified tissues (Figs. 6A, C) at a number of places along the root circumference very similar to the formation of lenticels (Figs. 5C, 6 D).

#### **4.4 Discussion**

In pomegranate roots, the Casparian strip formed at the exodermis earlier than the endodermis. It is a known that the endodermal Casparian strip blocks the free apoplastic passage of various ions, heavy metals and fluorescent dyes (Robards and Robb, 1972, Nagahashi et al., 1974). It is possible that the Casparian strip at exodermis may be functioning similarly, as a filter or barrier to apoplastic movement like the endodermis, in juvenile roots (Peterson et al., 1978, 1982; Peterson, 1987, Lehmann et al., 2000). Details of exodermis permeability have been reviewed by Hose et al., (2001). We have observed similar anatomy previously in roots of grapevine (Song et al., 2011b, Tuladhar and Nii, 2013), red bayberry (Song et al., 2011a) and feijoa (Nii et al., 2012) etc. It was concluded by Enstone and Peterson (1998) that endodermal Casparian strip was essential for root function whereas exodermal Casparian strips and suberin lamellae were only an adaptive feature. Enstone et al., (2003) have used the term 'physiological sheath' for exodermis and endodermis that play an important role in root function.

The endodermis is the youngest and the innermost cortical layer (Lux and Luxova, 2001). While tracking the developmental phases of pomegranate root tissues, endodermal maturity progressed up to stage III, that means endodermal cell walls thickened with lignin accumulation. So far there have been reports that the Casparian strip forms due to suberin accumulation in the cell wall of endodermal cells (Peterson, 1987, Peterson et al.,

1978, 1982) but Lux and Luxova (2001) have reported that the tertiary endodermis may become suberized or lignified. Lignin is a complex and highly variable biopolymer derived from the oxidative polymerization of cinnamyl alcohols bearing the three aromatic residues *p*-hydroxyphenyl, guaiacyl and syringyl (Freudenberg 1965, Whetten and Sederoff, 1995). When a chemical analysis was conducted in maize (*Zea mays* L.) the lignin composition of Casparian strips (primary endodermal cell walls) showed high syringyl content and was similar to lignin in secondary cell walls of the tertiary endodermal cell walls whereas lignin in secondary cell walls contained higher amounts of *p*-hydroxyphenyl units (Zeier et al., 1999). In the present study we witnessed the gradual accumulation of both suberin and lignin in endodermal cells (Figs. 3D-F) and in every new cell layer that formed beyond the endodermis (Fig. 4) but since the chemical analysis was not conducted in the present study the changes in lignin composition during the lignification process is unknown.

In our previous paper (Nii et al., 2012) we explained how polyderm layers form due to pericyclic cell division in feijoa (Myrtaceae) roots. Such regular accumulation of suberin and lignin was absent in pomegranate roots although it was clear that suberin accumulation preceded lignin accumulation at the endodermis (Figs. 3C, D). It is possible that lignin accumulation may be protecting the rapidly expanding endodermal walls from collapsing.

In the newly formed cells, though we could observe suberin accumulation, we can hardly conclude that they formed Casparian strips (Figs. 6 E, F) since it was totally different from endodermis-like cells in feijoa polyderm. Referring to Mager (1932), Bond (1930) and Mylius (1913), Lux and Luxova (2001) have mentioned that endodermal Casparian network density formed by Casparian bands, corresponds to each other in

neighboring cells. In another report by Meyer and Peterson (2011) multiseriate Casparian bands existed in the periderm of *Pelargonium hortorum* roots. From our studies on root maturation in grapevines, we reported that suberin accumulated in the periderm cells formed by pericyclic cell division (Song et al., 2011b, Tuladhar et.al, 2013).

In the present study, the newly formed cell layers consisted lignin accumulation, in addition to suberization that distinguished it from periderm observed in grapevine roots. On the other hand, lignin and suberin did not accumulate in separate cell layers like the polyderm in fejoa roots. They accumulated in the same cell layer. Therefore it was difficult to conclude whether the observed tissue may be called polyderm. Existence of Casparian strip and suberin accumulation has been reported in periderm (Meyer and Peterson, 2011; Tuladhar and Nii, 2013), however, lignin accumulation in periderm is uncommon. Alternative layers of lignin and suberin accumulating cell layers were reported in feijoa roots (Nii et al., 2012), however, in pomegranate roots even though lingo-suberic biopolymers could be detected, they did not exist in an alternating scheme.

In the present study, the width of the Casparian strip or suberin accumulating areas and lignified areas were not measured but the width of lignin in each cell was broader in mature roots. Suberin accumulation was visible in the cell wall of each cell that appeared to form a network. According to Lux and Luxova (2001), the role of the width of Casparian bands is related with the increased adherence of plasma membrane to the impregnated region of cell wall. Fahn (1964) has reported that the function of the network formed by Casparian bands as an apoplastic barrier was more effective when the protoplasts were attached to larger areas of the walls of the endodermal cells. Lignin deposit as *phi*-thickening, was associated with mechanical support by Mackenzie (1979). *Phi*-thickening was non-existent in pomegranate roots but large amounts of lignin-like

accumulation were seen in cell wall of every cell layer that formed due to cell division at phellogen. Important physiological properties of lignin include mechanical support, compressive strength, and plant defense based on its resistance to degradation (Campbell and Sederoff, 1996). The fact that so much lignin accumulated in outermost tissues of pomegranate roots may be contributing to tolerate and adapt to various soil environments. The biochemical formation of lignin and the anatomical aspects of the lignification process like distribution of the lignin in the cell wall and its effect on cell wall properties have not been completely established (Kutscha and Grey, 1970). Lignin research has gained steady momentum over the years and recently, an enzyme central to lignin biosynthetic pathway called caffeoyl shikimate esterase was described by Vanholme et al., (2013) that affects lignin deposit in *Arabidopsis thaliana*. Sachs et al., (1962) have reported that lignin inhibits biological attack of wood particularly by fungi and decreases wood's permeability to gases and liquids. Much needs to be uncovered about lignin accumulation in woody fruit tree roots.

The present study has been useful to uncover the prominent lignin accumulation in maturing pomegranate roots. However, further experiments are necessary to determine the effect of abiotic stress conditions like flooding or drought on the biopolymer accumulation in periderm layers and overall development of pomegranate root tissues. Also, root tissue density or tissue volume is a matter of prime interest anatomically and it is important when studying adaptive responses of plants to their environmental conditions. Since root cell wall is the first to be affected by any stress signal (Komatsu et al., 2010, Salvador et al., 2013), studies on cell wall modification and their function deserve more attention. In this study we uncovered a unique layout of lingo-suberic biopolymers in pomegranate roots from growth to maturity which may be a highly species specific

matter.

#### 4.5 Summary

Roots of pomegranate (*Punica granatum* L.), belonging to Punicaceae, were investigated anatomically to record changes in tissue development from growth to maturity. When roots start secondary growth, a protective tissue called polyderm composed of alternating suberized and non-suberized cell layers, develop beyond the endodermis in certain families of plants including Myrtaceae. Punicaceae is not known to develop a polyderm. However, new layers formed beyond the endodermis during secondary growth in pomegranate roots and biopolymer deposition was observed their cell walls. The present study aims to gather more knowledge on this tissue discovered in pomegranate roots and cross check whether it is a polyderm or a unique type of periderm.

Root specimens were sectioned freehand or with an ultramicrotome after embedding in Technovit 7100 resin. After staining root sections with berberine hemisulfate-aniline blue-safranin O series they were observed under fluorescent or optical microscopes.

Unlike the polyderm in Myrtaceae roots, in pomegranate roots, ligno-suberic material accumulated in every cell layer beyond the endodermis. The alternating suberized and non-suberized layers that define the polyderm were absent. Lignin accumulation in the cell wall was pronounced in every cell layer of this outermost tissue and suberin-like autofluorescence was also observed in the same layer. We considered this to be a unique feature typical in pomegranate periderm.

It is possible that accumulating both lignin and suberin in the same cell layer instead of alternative layers is more efficient because metabolic energy is not spent in forming a separate cell layer. Further experiments are underway to acknowledge changes in such

biopolymer accumulation in the outermost tissue in abiotic stress conditions.

#### 4.6 References

- Bond, G. 1930. The occurrence of cell division in the endodermis. Proc. Roy. Soc. Edinb. 50: 38-50.
- Brundrett, M.C., Enstone, D.E., Peterson, C.A. 1988. A berberine-aniline blue fluorescent staining procedure for suberin, lignin and callose in plant tissue. Protoplasma 146: 133–142.
- Campbell, M.M., Sederoff, R.R. 1996. Variations in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. Plant Physiol. 110: 3-13.
- Esau, K. 1970. Anatomy of Seed Plants. 2nd Edition. John Wiley and Sons, New York, pp. 185.
- Enstone, D.E., Peterson, C.A. 1998. Effects of exposure to humid air on epidermal viability and suberin deposition in maize (*Zea mays* L.) roots. Plant Cell and Env. 21: 837-844.
- Enstone, D.E., Peterson, C.A., Ma, F. 2003. Root endodermis and exodermis: structure, function and responses to the environment. J. Plant Growth Regul. 21:335-351.
- Fahn, A. 1964. Some anatomical adaptations of desert plants. Phytomorph. 14: 93-102.
- Freudenberg, K. 1965. Lignin: its constitution and formation from *p*-hydroxycinnamyl alcohols. Science 148: 595-600.
- von Guttenberg, H. 1968. Der primare Bau der Angiospermenwurzel. Handbuch der Pflanzenanatomie. Berlin/Stuggart: Gebruder Borntraegern 472.
- Hiwale, S.S., More, T.A., Bagle, B.G. 2011. Root distribution pattern in pomegranate

‘Ganesh’ (*Punica granatum* L.). Acta Hort. 890: 323-326.

Hose, E., Clarkson, D.T., Steudle, E., Schreiber, L., Hartung, W. 2001. The exodermis a variable apoplastic barrier. J. Exp. Bot. 52: 2245-2264.

Komatsu, S., Kobayashi, Y., Nishizawa, K., Nanjo, Y., Furukawa, K. 2010. Comparative proteomics analysis of differentially expressed proteins in soybean cell wall during flooding stress. Amino Acids 39: 1435-1449.

Kutscha, N.P., Grey, J.R. 1970. The Potential of Lignin Research. Technical Bulletin 41. Maine Agricultural Experiment Station. University of Maine, Orono, Maine. pp. 1-20.

Lehmann, H., Stelzer, R., Holzamer, S., Kunz, U., Gierth, M. 2000. Analytical electron microscopical investigations on the apoplastic pathways of lanthanum transport in barley roots. Planta 211: 816-822.

Luhan, M. 1955. Das Abschlussgewebe der Wurzeln unserer Alpenpflanzen. Berichte der Deutschen Botanischen Gesellschaft 68: 87-92

Lux, A., Luxova, M. 2001. Secondary dilation growth in the root endodermis. Recent Advances of Plant Root Structure and Function. Kluwer Academic Publishers, Netherlands. pp. 31-37.

Lux, A., Morita, S., Abe, J., Ito, K. 2005. An improved method for clearing and staining free-hand sections and whole-mount samples. Ann. Bot. 96: 986–996.

Mackenzie, K.A.D. 1979. The development of the endodermis and phi layer of apple roots. Protoplasma 100: 21-23.

Mager, M. 1932. Beitrage zu kenntnis de primaren wuzelrinde. Planta 16: 666-708.

Mann, C.E.T. 1930. Studies in the root and shoot growth of strawberry. The origin, development and function of roots of the cultivated strawberry (*Fragaria virginiana* x *chiloensis*). Ann. Bot. 44: 56-84.

- Meyer, C.J., Peterson, C.A. 2011. Casparian bands occur in the periderm of *Pelargonium hortorum* stem and root. *Ann. Bot.* 107: 591-598.
- Moerschbacher, B.M., Noll, U., Gorrichon, L., Reisener, H.J. 1900. Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. *Plant Physiol.* 93: 465-470.
- Mylius G 1913 Das Polyderm. *Bib. Bot.* 18: 1-119.
- Nagahashi, G., Thompson, W.W., Leonard, R.T. 1974. The Casparian strip as a barrier to the movement of lanthanum in corn roots. *Science* 183: 670-671.
- Nelson, P.E., Wilhelm, S. 1957. Some aspects of strawberry root. *Hilgardia* 26: 631-642.
- Nii, N., Ohtsuka, S., Ye, L., Song, Y. 2012. Formation of endodermis-like cells with Casparian strip and thick wall cells derived from pericycle in the roots of *Feijoa sellowiana* (Myrtaceae). *J. Japan. Soc. Hort. Sci.* 81: 314-319.
- Nii, N., Pan, C.X., Ogawa, Y., Cui, S.M. 2004. Anatomical features of the cell in-growth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. *J. Japan. Soc. Hort. Sci.* 73: 411-414.
- Pan, C.X., Nakao, Y., Nii, N. 2006. Anatomical development of *phi* thickening and the Casparian strip in loquat roots. *J. Japan. Soc. Hort. Sci.* 75: 445-449.
- Peterson, C.A. 1987. The exodermal Casparian band of onion blocks apoplastic movement of sulphate ions. *J. Exp. Bot.* 32: 2068-2081.
- Peterson, C.A., Peterson, R.L., Robards, A.W. 1978. A correlated histochemical and ultrastructural study of the epidermis and hypodermis of onion roots. *Protoplasma* 96: 1-21.
- Peterson, C.A., Emanuel, M.E., Wilson, C. 1982. Identification of a Casparian band in

the hypodermis of onion and corn roots. Can. J. Bot. 60: 1529-1535.

Robards, A.W., Robb, M.E. 1972. The entry of ions and molecules into roots: an investigation using electron-opaque tracers. Planta 120: 1-12.

Sachs, I., Clark, I., Pew, J. 1962. Techniques to locate lignin in wood cells. Chem and Eng. News. 40: 41-42.

Salvador, V.H., Lima, R.B., Dos Santos, W.D., Soares, A.R., Bohm, P.A.F., Marchiosi, R., Ferrarese, M., Ferrarese-Filho, O. 2013. Cinnamic acid increases lignin production and inhibits soybean root growth. PLoS ONE 8(7): e69105. doi:10.1371/journal.pone.0069105.

Song, Y., Ye, L., Nii, N. 2011a. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Hortic. 129: 554–560.

Song, Y., Ye, L., Tuladhar, A., Nii, N. 2011b. Anatomical development of cell structure including Casparian strip during root growth in grapevines. J. Japan. Soc. Hort. Sci. 80: 164-168.

Teixeira da Silva, J.A., Rana, T.S., Narzy, D., Verma, N., Meshram, D.T., Ranade, S.A. 2013. Pomegranate biology and biotechnology: A review. Sci. Hortic. 160: 85-107.

Tripathi, S.M., Singh, D.K. 2000. Molluscicidal activity of *Punica granatum* bark & *Canna indica* root. Braz. J. Med. Biol. Res. 33: 1351-1355.

Tuladhar, A., Nii, N. 2013. Anatomical changes of root thickening and shedding process of outer layers during secondary growth in grapevine roots. Sci. Rep. Fac. Agri. Meijo Univ. 49: 11-17. (in Japanese with English abstract).

Tuladhar, A., Ohtsuka, S., Nii, N. 2014. Formation of exclusive pattern during accumulation of lingo-suberic material in cell wall of Myrtaceae root tissues including

epidermis, exodermis, endodermis and polyderm. Plant Root 8: 24-32.

Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goemine, G., Kin, H., Cross, J., Morreel, K., Araujo, P., Welsh, L., Hastraete, J., McClellan, C., Vanholme, B., Ralph, J., Simpson, G., Halpin, C., Boejan, W. 2013. Caffeoyl Shikimate Esterase (CSE) is an enzyme in the lignin biosynthetic pathway in Arabidopsis. Science 341: 1103.

Whetten, R., Sederoff, L. 1995. Lignin biosynthesis. Plant Cell 7: 1001-1013.

Williamson, B. 1984. Polyderm, a barrier to infection of red raspberry buds by *Didymella applanata* and *Botrytis Cinerea*. Ann. Bot. 53: 83-89.

Zeier, J., Ruel, K., Ryser, U., Schreiber, L. 1999. Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/ rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. Planta 209: 1-12.

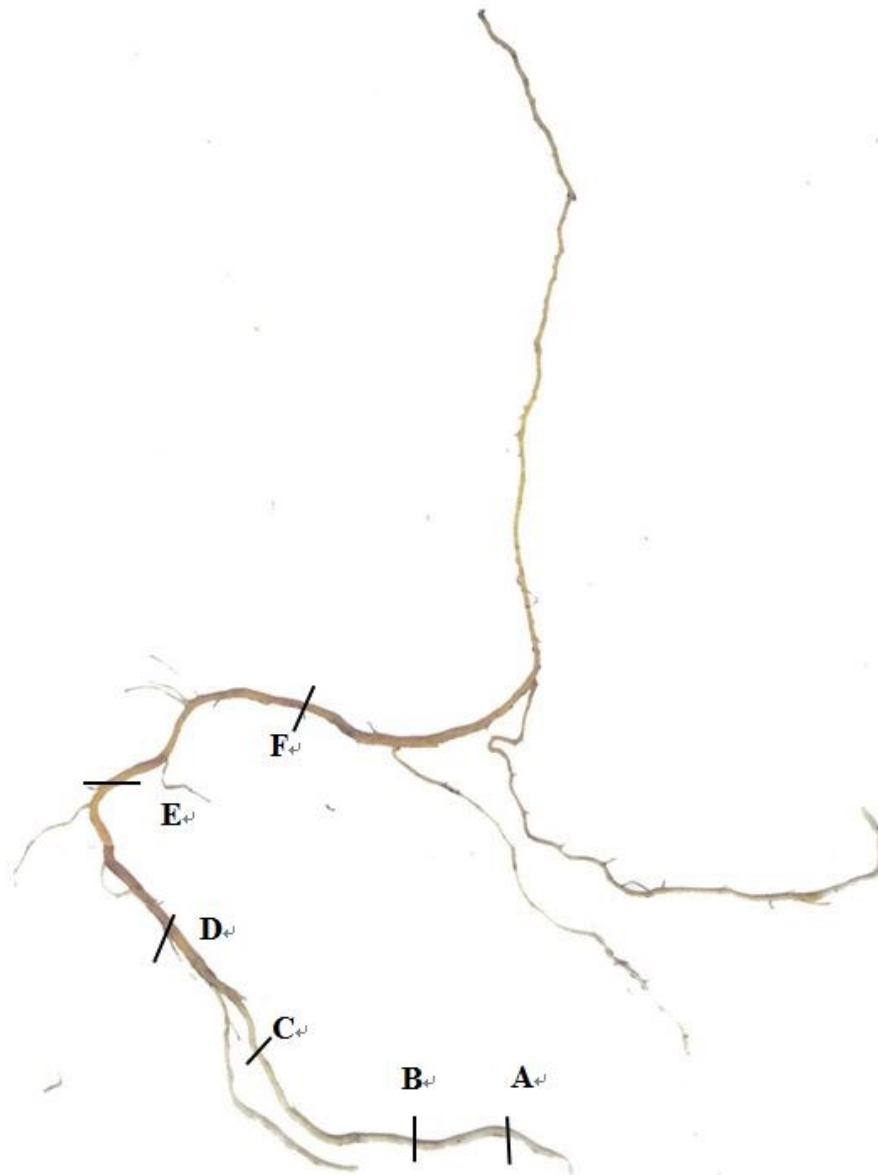


Fig.4-1. A scanned view of representative pomegranate main root illustrating growth from new white roots to old, brown pomegranate roots. The markings, A-F, indicate the area where cross-sections in Figs. 2 A-F were cut at a distance of 10, 20, 60, 80, 110, 140 mm, respectively. Cross-sections of Figs. 3 A-F were also prepared from the same root but panel names and distance from the root tip are named differently.

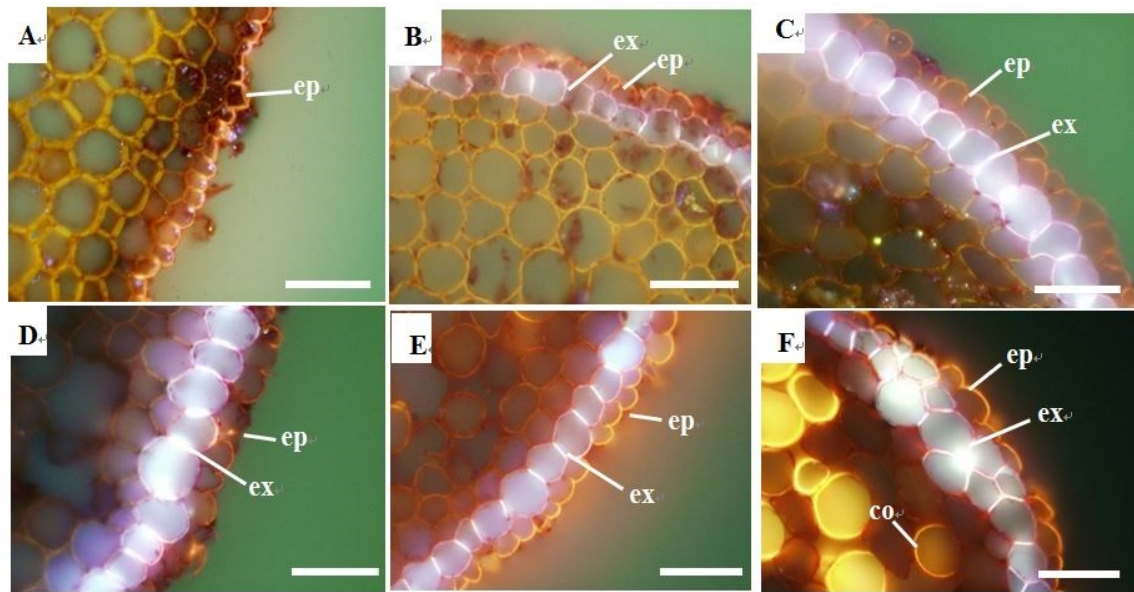


Fig.4-2. Lignin-like deposit in the rhizodermis and suberin-like deposit and Casparian strip formation at the exodermis in relation to pomegranate root age. Root samples were stained with berberine hemisulfate-aniline blue-safranin O. Lignin encrusted rhizodermis turned reddish orange under the fluorescent microscope and suberized areas in exodermis appeared milky white. Casparian strip formed in one or more layers at the exodermis. Distance from the root tip, panel A: 10 mm, B: 20 mm, C: 60 mm, D: 80 mm, E: 110cm, F: 140 mm. ep: rhizodermis/ epidermis; ex: exodermis; co: lignified cortical cell. Scale = 50 μm.

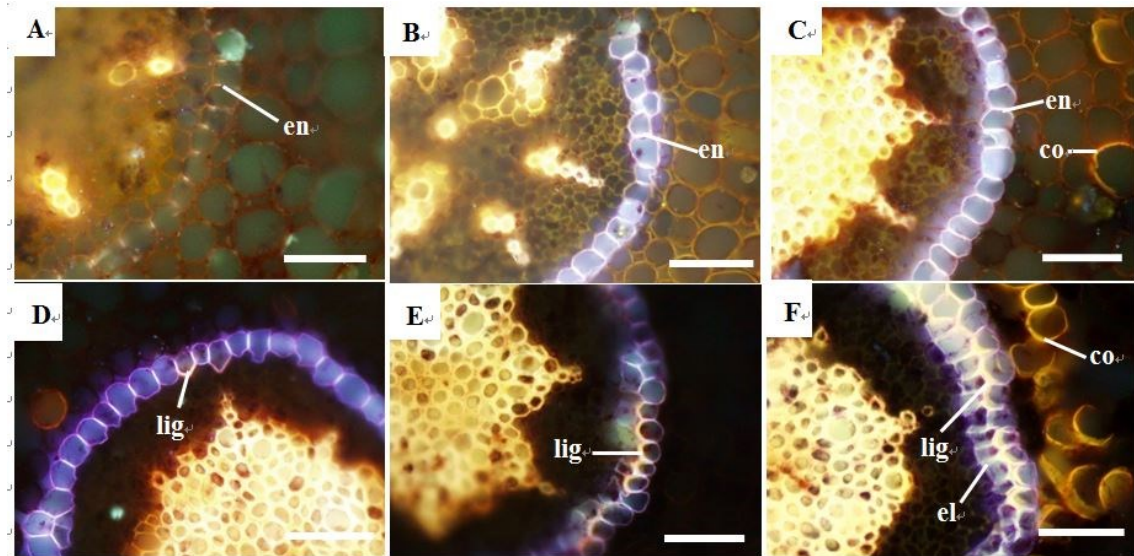


Fig.4-3. Suberin accumulation and development of Casparian strips in the endodermis and formation of the first new layer with lignin-like accumulation beyond the endodermis in pomegranate roots. Root samples were stained with berberine hemisulfate-aniline blue-safranin O. Lignin encrusted cortical cells and few endodermal cells appear reddish orange. Distance from the root tip, panel A: 20 mm, B: 60 mm, C: 80 mm, D: 110 mm, E: 130 mm, F: 150 mm. co: lignified cortical cell, en: endodermis, el: endodermis-like cells, lig: lignin accumulation. Scale = 25 $\mu$ m.

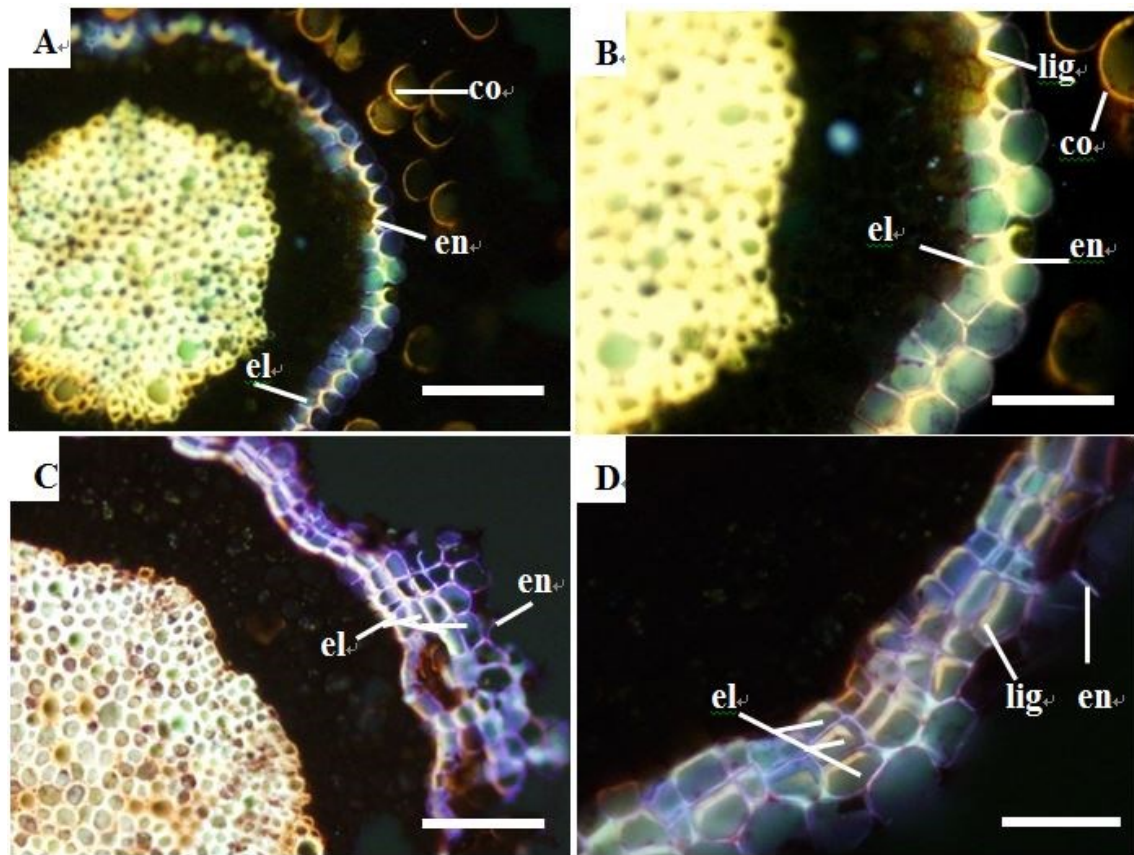


Fig.4-4. Lignin-like accumulation at the endodermis and formation of new cell layers beyond the endodermis in pomegranate roots. Root samples were stained with berberine hemisulfate-aniline blue-safranin O. Lignified cell walls appear to be reddish orange and suberized layers appear to be milky white under the fluorescence microscopy. Distance from the root tip, panel A: 130 mm, C: 150 mm; B, D are enlargements of A, C, respectively. en: endodermis, el: endodermis-like cells, lig: lignin accumulation, co: lignified cortical cell. Scale = 50 $\mu$ m (Panels B, D); 100 $\mu$ m (Panels A, C).

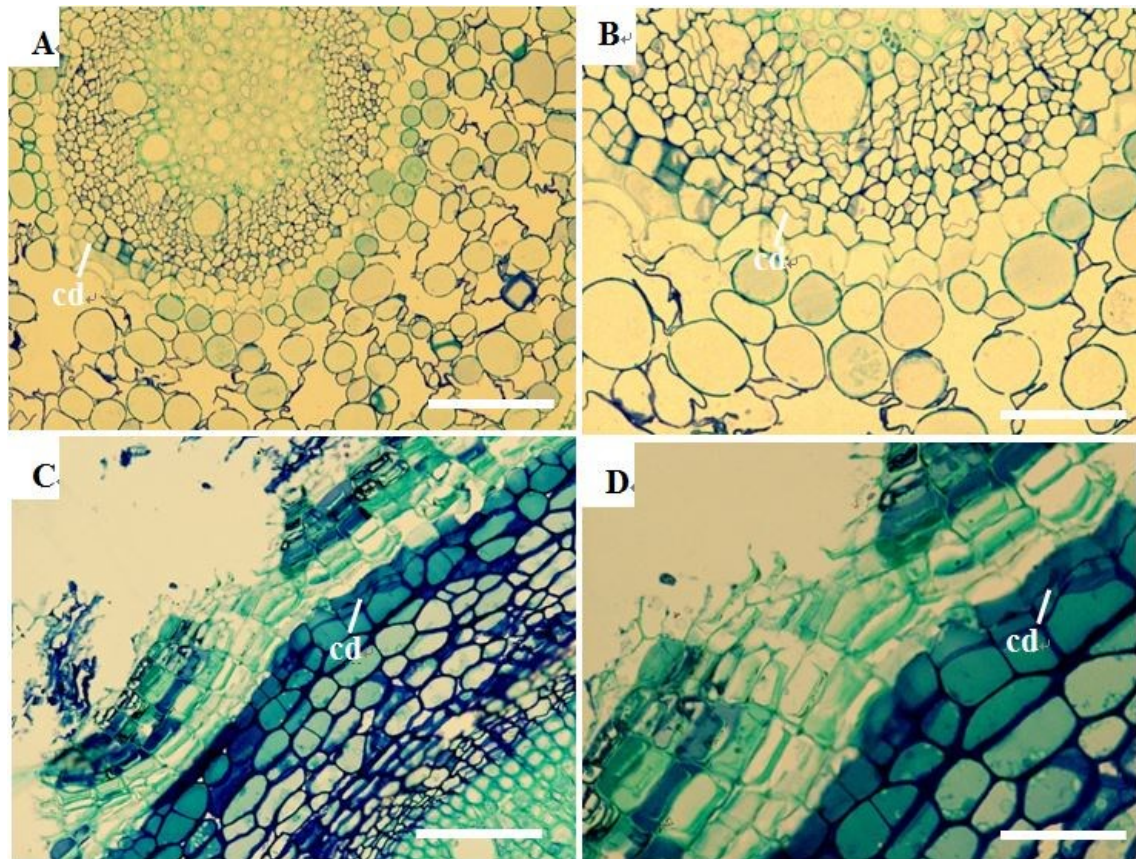


Fig.4-5. Cell divisions occurring in pericycle and phellogen of pomegranate root. Older root samples different from Figs.2-4 were embedded in Technovit resin and stained with methylene blue. One or more tangential plates appeared in the cells at the pericycle and phellogen which caused cell division. Distance from the root tip, panel A: 80 mm, C: 110 mm; B, D are enlargements of A, C, respectively. cd: cell division. Scale = 50 $\mu$ m (Panels B, D); 100 $\mu$ m (Panels A, C).

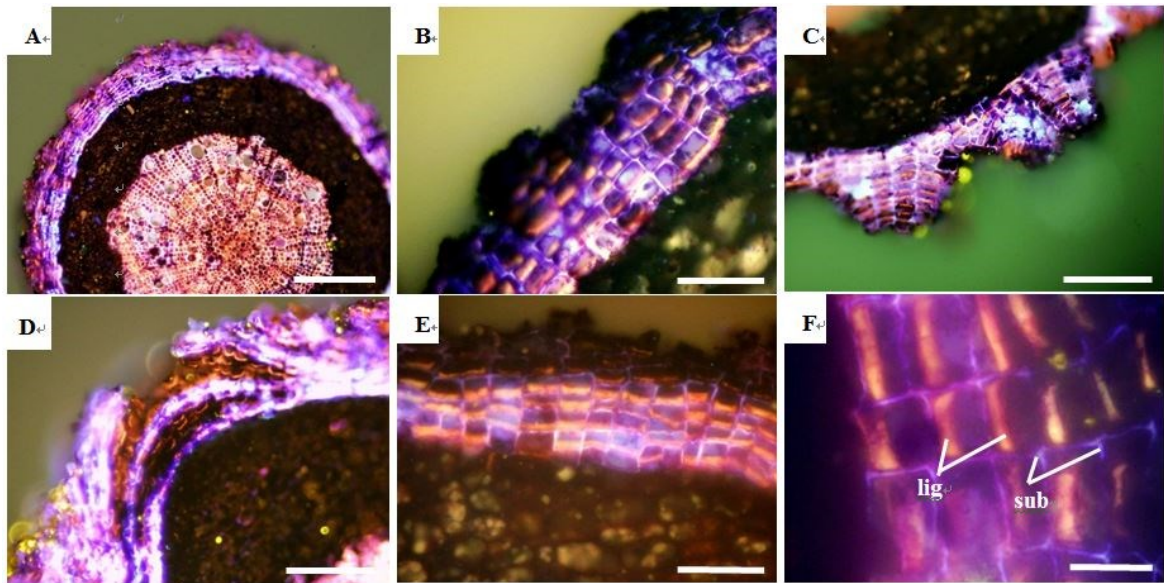


Fig.4-6. Photomicrographs of cross-sections of pomegranate roots showing the formation of lignified cell layers at the periderm. Panel A shows multiple cell layers that are the outermost layers after endodermis, cortex, exodermis and rhizodermis shed off. Panel B is enlargement of panel A. Panel C shows triangular chunks of lignified and suberized periderm layers with openings in the middle. Panel D shows lenticel-like increase in thick-walled lignified cells at the root surface. Panel E shows lignin-like accumulation which is “closed” centripetally and suberin-like accumulation in the cell wall resembling a network. Panel F is enlargement of panel E. Distance from the root tip, panel A, B: 150 mm, C: 160 mm, D, E, F: 170 mm lig: lignin-like substances accumulating layer; sub: suberin-like substances accumulating layer. Scale = 25 $\mu$ m (Panel F); 50  $\mu$ m (Panel B, C, E); 100 $\mu$ m (Panel D); 500  $\mu$ m (Panel A).

## **Chapter 5**

**Anatomical study on *phi*-thickening and intercellular suberin accumulation in root cortex of loquat (*Eriobotrya japonica* Lindl.) grown in different environmental conditions**

## 5.1 Introduction

*Phi*-thickening forms due to cell wall ingrowth that becomes impregnated with lignin and is named so because it resembles the Greek letter “φ” in cross-section (Russow, 1875 as quoted by Gerrath et al., 2002). It is considered to be a genetic feature of certain plant species and so there have been efforts to apply it as a systematic characteristic (Gerrath et al., 2002). However, relying on *phi*-thickening was considered unsuitable for this purpose because plants that developed *phi*-thickening under normal condition failed to do so when grown *in-vitro* (Soukup et al., 2004). Do growth conditions affect *phi* development? If so, what is the role of *phi*-thickening? Unlike the Casparian strip, role of *phi*-thickening has not yet been established. Mackenzie (1979) proposed that *phi*-thickening may be providing mechanical support to roots growing in dry soil. However, it has never been demonstrated. So far, detailed studies on *phi*-thickening in different growth stress conditions are still very limited. In our previous study on loquat (*Eriobotrya japonica* Lindl.) roots, we reported that *phi*-thickenings increased considerably under drought stress condition (Pan et al., 2006). There was another report by Fernandez-Garcia et al., (2009) that *phi*-thickenings increased in *Brassica oleraceae* under salt stress. We felt the need to conduct more investigations on development of *phi*-thickenings under various other growth stress conditions to fully understand its role. In the present study, loquat roots grown under water-logged condition were studied. In addition, there is very limited knowledge on *phi*-development in radicles. In this study, loquat seeds were germinated under extraordinary conditions, like low temperature inside a refrigerator and in a dark cabinet test tubes filled with distilled water were also examined.

*Phi*-thickening has been previously observed in other Rosaceae fruit trees like

pears (*Pyrus communis* L.) by Esau (1943), apples (*Malus pumila* Mill.) by Mackenzie (1979) and sweet cherry (*Prunus avium* L.) by Soukup et al., (2004). Loquat belongs to the same Rosaceae family. *Phi* formation in loquat was most pronounced when compared with a few other Rosaceae fruit trees (Pan and Nii, 2005). Therefore, loquat is an important species for studying *phi*-thickening.

In their report, Gerrath et al., (2002) have mentioned that Van Tieghem (1888) first classified *phi*-thickening into three types based on their location in the root cortex. The first and most common pattern of *phi*-thickening is in the cortical cell layers close to the endodermis (type I), the second distribution pattern is in the cortical layers close the epidermis (type II) and *phi*-thickening found in one or more layers throughout the cortex is the third type (type III). According to Van Tieghem (1888), the type of *phi*-thickening was generally consistent for a given species, although some species exhibited more than one distribution pattern. In loquats, we confirmed the presence of type I *phi*-thickening and the absence of type III *phi*-thickening (unpublished data). However in cortical cell layers close to the epidermis, biopolymer accumulation was observed. We confirmed that they were not type II *phi*-thickening firstly because they appeared in the intercellular space between cortex cells rather than in adjacent cell walls. Secondly, they did not resemble the Greek letter “phi.” Thirdly, they turned red when stained with Sudan red, implying suberin accumulation but didn’t turn red when stained with phloroglucinol- HCL implying almost no lignin accumulation. In this study we re-named this accumulation of suberin as intercellular suberin accumulation (ISA). In our previous report (Pan et al., 2006), ISA was not taken into account. In the present report, effect of growth conditions was examined in ISA as well. Following our three previous publications (Nii et al., 2004; Pan and Nii, 2005; Pan et al., 2006) on

*phi*-thickenings of Rosaceae fruit trees, the present investigation is a comparative study on *phi* development and ISA in both seed germinated roots and tree roots grown in different soil water conditions including extraordinary conditions. Studying anatomical changes occurring in root tissues is important to elucidate the role of biopolymer accumulation in plant survival under unfavorable growth condition.

## 5.2 Materials and Methods

Roots of two-year-old grafted loquat (*Eriobotrya japonica* Lindl.) trees (cultivar ‘Mogi’) and radicle germinated from seeds of the same cultivar were used for this investigation. The young trees were planted in standard ‘Wagner’ pots (1/5000 a) filled with sandy soil and placed in a greenhouse. Six pots were allotted for each treatment group and drought, water-logged and normal treatments were maintained for about four months from May to August, 2013. Pots in the water-logged treatment were watered every day until it saturated the soil and leaked out to collect in plastic dishes placed under the pots. Pots in the normal treatment were watered when the soil surface became dry. Pots in the dry treatment were not watered until the leaves began to wilt.

The extent of stress from the treatments was estimated by measuring the water saturated deficiency (WSD) of leaves. Two leaves were collected from each treatment before watering. The leaves were cut at the basal end of the petiole and field fresh weight of leaves was recorded immediately. Then after immersing them in distilled water under saturated moist conditions, the saturated fresh weight was measured after 24 hours. The dry weight was determined after lyophilization (lyophilized dry weight). WSD was calculated, based on fresh weight and dry weight from the following equation. The result of WSD, was significant according to Tukey’s multiple test range and effect

of treatment conditions can be observed in Table 1.

$$\frac{(\text{saturated fresh weight} - \text{field fresh weight})}{(\text{saturated fresh weight} - \text{lyophilized dry weight})} \times 100 (\%)$$

Before starting the treatment, new white roots were removed from each tree so that only the roots grown during the treatment period were used for observation. At the end of treatment period, newly grown white roots were collected from all three treatment groups for anatomical observation. Transverse sections were cut by hand using a stainless blade at a distance of 5 mm, 15 mm, 25 mm, 35 mm from the root tip then observed under a BX60 optical/ultraviolet microscope (Olympus, Tokyo, Japan) (excitement wavelength: 365 nm) without staining or after staining with phloroglucinol-HCl for lignin and Sudan red 7B for suberin (Fig.1). Formation of *phi*-thickening was compared between 5 to 35 mm from the root tip. The extent of *phi* formation and intercellular suberin accumulation (ISA) in cortical cell layers were classified into 4 grades as shown in Fig. 2.

This was also examined in roots germinated in extraordinary conditions. Seeds of fresh loquat fruits were collected and germinated under 3 different conditions: 1) in plastic pots (diameter 6.5 cm, depth 5.5 cm) filled with sandy soil inside a greenhouse, 2) in moist plastic bags placed in a refrigerator at 4°C, 3) in test-tubes filled with distilled water under lightless condition at room temperature. Water in the test-tubes was changed every few days. When roots were about 40 mm they were hand-sectioned and observed following the same procedure mentioned above.

### 5.3 Results

*Formation of phi- thickening in cortical cells adjacent to the endodermis*

In grafted trees, though *phi*-thickening was observed in all treatments, cortical cell layers developing *phi*-thickening differed among treatment groups as shown in Table 2. 14% of roots grown in drought condition developed up to 4 layers of *phi*-thickening but none of the roots grown in water-logged and normal condition developed as many layers of *phi*-thickening. 18% of roots grown in water-logged condition developed only 1 layer of *phi*-thickening. 71% of roots grown in water-logged condition developed 2 layers of *phi*-thickening. Under drought condition, roots with only 1 layer of *phi*-thickening did not exist. In normally treated roots, 50% developed 2 layers and other 50% developed 3 layers of *phi*-thickening. None of the roots grown in normal condition developed 1 or 4 layers. It was concluded that in grafted trees, *phi*-thickening increased up to 4 layers under drought conditions but did not increase more than 2-3 layers in water-logged or normal conditions.

In radicles too, *phi*-thickening was observed in all treatment conditions but cortical cell layers developing *phi*-thickening varied according to the growth conditions as shown in Table 3. 100% of radicles in refrigerator at 4°C and in water-filled test tubes developed only 1 layer of *phi*-thickening. 66% of radicles in sandy soil developed 2 layers of *phi*-thickening and 25% developed up to 3 layers. None of the radicles developed 4 layers of *phi*-thickenings. It was concluded that the formation of *phi*-thickening got restricted in radicles in moist low temperature and distilled water than radicles grown in regularly watered sandy soil.

#### *Formation of intercellular suberin accumulation (ISA) in cortical cells near the epidermis*

In grafted trees, all roots developed ISA in cortical cell layers near the epidermis

though the number of cell layers developing it varied as shown in Table 2. 24% of roots grown in drought condition, developed up to 4 layers of ISA. In water-logged roots, 2 layers of ISA developed in 88% of the roots. In normal growth condition, 80% of roots developed 3 layers of ISA. Roots with only 1 layer were observed in 6% of water-logged roots. Water-logging restricted ISA whereas drought condition had the opposite effect.

In radicles, ISA did not develop up to 4 layers (Table 3). 100% of radicles germinated in distilled water developed only 1 layer of ISA. In moist low temperature, 93% of radicles developed 2 layers of ISA. In sandy soil, 67% of roots developed 3 layers of ISA. Germination in sandy soil was comparatively drier than moist conditions inside a refrigerator. It was concluded that in radicles, ISA decreased in excess moisture and low temperature but increased in drought conditions. It is important to note that 100% of roots germinated in distilled water developed 1 layer of *phi*-thickening and 1 layer of ISA.

Both *phi*-thickenings and ISA appeared in loquat roots before the formation of the Casparian strip at the endodermis (Fig.3A). As the distance from the root tip increased, endodermal roots suberized gradually (Fig. 3 B).

## 5.4 Discussion

In the present study, development of *phi*-thickening and ISA were compared in roots of grafted loquat trees and radicles under different growth conditions. Comparison was made in root cross-section prepared at 35 mm from the root tip when roots were undergoing primary growth. *Phi*-thickenings are known to exist only during primary growth (Weerdenburg and Peterson 1983). *Phi*-thickenings and ISA started developing

from a distance of 5 mm from the root tip and developed to maximum capacity at 35 mm. *Phi*-thickenings and ISA did not increase further beyond the distance of 35 mm so comparison was made at 35 mm from the root tip.

Despite the different treatments, *phi*-thickening were present in all treatments suggesting that it is a genetic trait in loquats. In Cupressaceae and Pinaceae, Gerrath et al. (2005) reported that although root morphology and *phi*-thickening were significantly affected by the environment, the presence of *phi*-thickening itself was not, so it is evidently a constitutive anatomical feature in the examined species. Pan and Nii (2005) observed that the extent of *phi*-thickening formation differed among four Rosaceae fruit trees including loquat and this could be a species-specific feature. From the present examination it is evident that exposure to water-logging and extraordinary conditions caused significant decrease in *phi* formation as well as ISA in the root cortex. Soukup et al. (2004) have reported that the formation of *phi* -thickening was avoided when wild cherry (*Prunus avium*) was grown *in vitro*. These studies have shown that *phi*-development is not necessarily constitutive.

Our results in the present study show that both *phi*-thickenings and ISA increased under drought conditions and decreased under water-logged and extraordinary conditions. The exodermal Casparian strip was not observed but suberin accumulated in the intracellular space of cortical cells before the suberization of endodermis cells (Fig. 3A). *Phi*-thickenings and ISA were observed before the endodermal cells suberized so they may be playing a physiological role in root function like the Casparian strip (Fig.3B). However, endodermal cells are characterized by the presence of suberin in the Casparian strips, while *phi*-thickenings consist of lignin deposits (Millay et al. 1987).

Regarding the permeability properties of *phi*-thickenings, apple and geranium roots

were tested by Peterson et al. (1981). They used fabric brightener dye which did not penetrate the endodermal layers but penetrated the *phi*-thickenings proving that *phi*-thickenings do not function as barriers to the apoplastic transport of small molecules of dye. In a more recent examination by Lopez-Perez et al., (2007) who studied broccoli roots under salt stress, it was reported that *phi*-thickenings could be a partial physical barrier only to apoplastic water transport. According to their report, *phi*-thickenings were more numerous in plants grown in higher concentration of NaCl than in non-stressed plants.

Since its discovery in the 19<sup>th</sup> century, the role of *phi* -thickening has been a subject of much speculation and experimentation. So far, the only established argument is of mechanical support (Mackenzie, 1979). The fact that *phi*-thickening increased under drought stress in loquats (Pan et al., 2006) suggests that *phi*-shaped accumulation of lignin in the cell wall could be necessary for young root tips to withstand and penetrate through dry, hard soil. Lignin is a polymer of phenol propane units that accumulates within the cellulose strands and toughens the cell wall (Cutter, 1970). Perhaps in moist soil, there is less need for cells to accumulate lignin for mechanical support unlike dry soil. *Phi*-thickenings increased in higher concentration of salt in *Brassica oleracea* (Fernandez-Garcia et al., 2009) and *phi*-thickenings were detected in *Zea mays* only when grown in solid-waste slag (Degenhardt and Gimmler, 2000). Somehow soil substrate seems to be an important parameter that determines *phi*-development in roots. What mechanisms control site-specific lignin accumulation in the shape of *phi* as a response to soil environment in inner cortex and suberin accumulation in intercellular space in the outer cortex? It is known that plant cells can modify their cell walls in many ways with subcellular precision but much remains unknown about mechanism

that controls such site-specific cell wall modification at molecular level (Roppolo and Geldner, 2012).

In this investigation, *phi*-thickening developed in 2 to 3 layers in water-logged and normal treatments whereas in roots grown in drought condition, it increased up to 4 layers in loquat tree roots. It was clear that treatment conditions affected not only *phi*-thickening but also ISA similarly. When *phi*-thickening increased, ISA also increased under drought condition and when *phi*-thickening decreased, ISA also decreased under water-logged condition. It seems there is a relation between lignin and suberin accumulation although they are different biochemically and synthesized differently by plants (Fig.1).

Germination in low temperature and distilled water restricted the *phi*-development and ISA in radicles that are most sensitive to growth environment as well.

It is also important to consider that *phi*-thickenings and ISA existed until the cortex was intact to the stele in primary roots. In other words, newly growing root tips are most sensitive to its environment and *phi* formation and ISA are traceable responses in loquats. Since *phi*-thickening and ISA slough off with the cortex as roots mature, it is clear that *phi*-thickening and ISA have no role in secondary growth of roots. In mature roots of loquat undergoing secondary growth, several layers of periderm became the outermost layers after cortex sloughed off and suberin accumulation was observed in the cell wall of periderm layers (unpublished data). Deposition of biopolymers like lignin and suberin in cell wall of cortex cells seems to be flexible according to growth environment and root age. In a review specializing on root cortex by Lux et al., (2004) it is mentioned that cortex might be designated as a root buffer zone, especially under stress conditions. According to Lux et al., (2004), cortex is the site for reserve material

deposition, aerenchyma formation, location for symbiosis and even photosynthesis including biopolymer accumulation. From our results, it is evident that a flexible mechanism controls the site-specific *phi*-shaped lignin accumulation and intercellular suberin accumulation in the cortex near the root tips and a positive correlation seems to exist between lignin (*phi*-thickening) and suberin (ISA) accumulation. This novel finding is important to understand the structural and functional variation of root cortex as it responds to environmental stress.

## 5.5 Summary

Roots of two-year-old grafted ‘Mogi’ trees and radicles germinated from seeds were investigated to determine whether *phi*-formation in loquat roots is genetic in nature or affected by different environmental conditions.

The grafted trees were grown under water-logged, drought and normal conditions and the seeds were germinated in sandy soil, in distilled water at room temperature and at 4°C. The extent of *phi*-thickening was compared at 5 to 35 mm from the root tip under fluorescent or optical microscopy after staining with phloroglucinol-HCL and Sudan red.

In all treatments, *phi*-thickening was observed in cortex cells adjacent to the endodermis and suberin accumulation was observed in intercellular space of cortex cells near the epidermis. Both *phi*-thickening and intercellular suberin accumulation (ISA) were affected by different treatments. They decreased under water-logged condition and increased under drought condition.

*Phi*-thickening and ISA can be considered a species-specific feature of loquat roots. However, growth conditions affected the extent of their development. Biopolymers like

lignin and suberin play important roles in enhancing root survival under stressful growth conditions. It is important to gain further knowledge on the mechanisms that control biopolymer deposition in root tissues.

## **5.6 References:**

Cutter, E.G. 1970. Plant Anatomy: Experiment and Interpretation. Edward Arnold, London.

Degenhardt, B., Gimmler, H. 2000. Cell wall adaptations to multiple environmental stresses in maize roots. J. Exp. Bot. 51:595-603.

Esau, K. 1943. Vascular differentiation in the pear root. Hilgardia 15:299-324.

Fernandez-Garcia, N., Lopez-Perez, L., Hernandez, M., Olmos, E. 2009. Role of phi cells and the endodermis under salt stress in *Brassica oleraceae*. New Phytol. 181:347-360.

Gerrath, J.M., Covington, L., Doubt, J., Larson, D. 2002. Occurrence of phi thickenings is correlated with gymnosperm systematics. Can. J. Bot. 80:852-860.

Gerrath, J.M., Matthes, U., Purich, M., Larson, D. 2005. Root environmental effects on phi thickening production and root morphology in three gymnosperms. Can. J. Bot. 83:379-385.

Lopez-Perez, L., Fernandez-Garcia, N., Oimos, E., Carvajal, M. 2007. The phi thickening in roots of broccoli plants: An acclimation mechanism to salinity? Int. J. Plant Sci. 168:1141-1149.

Lux, A., Luxova, M., Abe, J., Morita, S. 2004. Root cortex: structural and functional variability and responses to environmental stress. Root research 13: 117-131.

Mackenzie, K.A.D. 1979. The development of the endodermis and Phi layer of apple roots. Protoplasma 100:21-32.

Millay, M.A., Taylor, T.N., Taylor, E.L. 1987. Phi thickenings in fossil seed plants from Antarctica. IAWA Bulletin 8:191-201.

Nii, N., Pan, C.X., Ogawa, Y., Cui, S.M. 2004. Anatomical features of cell wall ingrowth in the cortical cells outside the endodermis and the development of the Casparian strip in loquat trees. J. Japan Soc. Hort. Sci. 73:411-414.

Pan, C.X., Nii, N. 2005. Anatomical development of phi-thickening in the roots of Rosaceae fruit trees. Sci. Rept. Fac. Agri. Meijo. Univ. 41:7-13.

Pan, C.X., Nakao, Y., Nii, N. 2006. Anatomical development of *phi*- thickening in and the Casparian strip in loquat roots. J. Japan. Soc. Hort. Sci. 75:445-449.

Peterson, C.A., Emanuel, M.E., Weedenburg, C.A. 1981. The permeability of phi thickenings in apple (*Pyrus malus*) and geranium (*Pelargonium hortorum*) roots to an apoplastic fluorescent dye tracer. Can. J. Bot. 59: 1107-1110.

Roppolo, D., Geldner, N. 2012. Membrane and walls: who is master, who is servant? Curr. Opin. Plant Biol. 15:608-617.

Russow, E. 1875. Betrachtungen uber das Leitbündel-und Grundgewebe aus vergleichend morphologischem und phylogenetischem Gesichtspunkt. Schakenburg's Anstalt, Dorpat (Quoted from Fernandez-Garcia N, Lopez L, Hernandez M, Olmos E (2009) Role of phi cells and the endodermis under salt stress in *Brassica oleracea*. New Phytol. 181:347-360).

Soukup, A., Mala, J., Hrubcova, M., Kalal, J., Votruba, O. 2004. Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry- tree plantlets during acclimation. Biol. Plantarum. 48:481-489.

Van Tieghem, P. 1888. Le reseau de soutien de l'écorce de la racine. Ann. Sci. Nat. Bot. Ser. 7/8: 375–378. (Quoted from Gerrath, JM, Covington L, Doubt J, Larson D (2002) Occurrence of phi thickenings is correlated with gymnosperm systematics. Can. J. Bot. 80: 852-860).

Weerdenburg, C.A., Peterson, C.A. 1983. Structural changes in phi thickening during primary and secondary growth in roots. 1. Apple (*Pyrus Malus*) Rosaceae. Can. J. Bot.

61:2570-2576.

Table. 5-1. WSD% of leaves in two-year-old loquats grown under water-logged, drought and normal treatments

| Treatment    | WSD (%)            |
|--------------|--------------------|
| Water-logged | 3.8 a <sup>z</sup> |
| Drought      | 12.3 c             |
| Normal       | 7.8 b              |

<sup>z</sup>Different letters are significantly different by Tukey's multiple range test (P <0.01).

Table.5- 2. The development of *phi*-thickening and intercellular suberin accumulation in two-year-old grafted loquat roots under water-logged, drought and normal treatments

| Treatment                                | <i>Phi</i> -thickening |    |     |      | Intercellular suberin accumulation |    |     |      |
|------------------------------------------|------------------------|----|-----|------|------------------------------------|----|-----|------|
|                                          | + <sup>z</sup>         | ++ | +++ | ++++ | +                                  | ++ | +++ | ++++ |
| Water-logged (n=17 <sup>y</sup> )<br>(%) | 18                     | 71 | 12  | 0    | 6                                  | 88 | 6   | 0    |
| Drought (n=21)<br>(%)                    | 0                      | 38 | 43  | 14   | 0                                  | 38 | 38  | 24   |
| Normal (n=10)<br>(%)                     | 0                      | 50 | 50  | 0    | 0                                  | 20 | 80  | 0    |

<sup>y</sup>: number of roots used for experiment.

+<sup>z</sup>, ++, +++, ++++: *phi*-thickening layers appeared in one layer, two layers, three layers, and more than four layers of the cortex, respectively. The root sections used for examination were observed at 35 mm behind the root tip.

Table.5-3. The development of *phi* – thickening and intercellular thickening in seed germinated roots grown under different treatments

| Treatment                       | <i>Phi</i> -thickening |    |     | Intercellular suberin accumulation |    |     |
|---------------------------------|------------------------|----|-----|------------------------------------|----|-----|
|                                 | + <sup>z</sup>         | ++ | +++ | +                                  | ++ | +++ |
| 4°C (n=14 <sup>y</sup> )<br>(%) | 100                    | 0  | 0   | 7                                  | 93 | 0   |
| Water (n=15)<br>(%)             | 100                    | 0  | 0   | 100                                | 0  | 0   |
| Sandy Soil (n=12)<br>(%)        | 8                      | 66 | 25  | 0                                  | 33 | 67  |

<sup>y</sup>: Number of roots used for measurement.

+<sup>z</sup>, ++, +++, ++++: *phi*-thickening layers appeared in one layer, two layers, three layers, and more than four layers of the cortex, respectively. The root sections used for examination were observed at 35 mm behind the root tip.

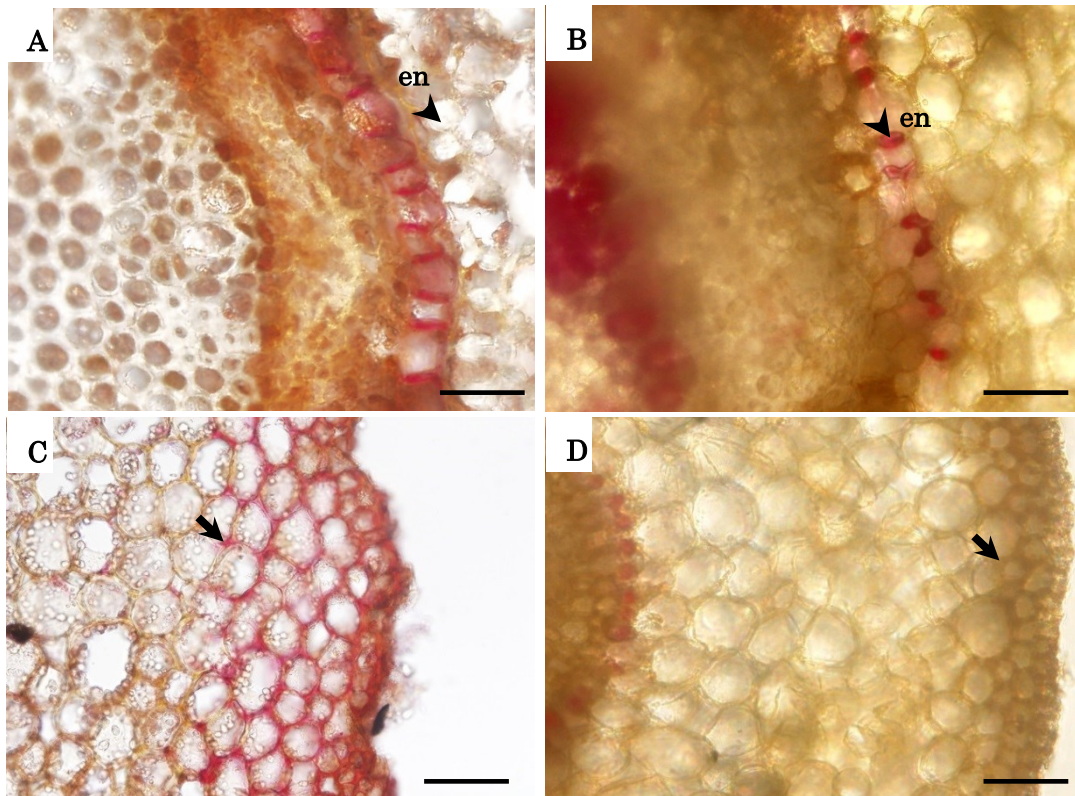


Fig.5-1: Result of staining with phloroglucinol-HCL and Sudan red. Panels A, C were stained with Sudan red and panels B, D were stained with phloroglucinol-HCL. Suberin accumulation was observed in endodermis with the Casparian strip and intercellular space between cortical cells near the epidermis. Lignin accumulation was observed in *phi*-thickening and xylem but not in intercellular space between cortical cells near the epidermis. Black arrowheads show *phi*-thickening on the cortical cell layer adjacent to the endodermis. Black arrows show intracellular space between cortical cells near the epidermis. en: endodermis; x: xylem. Scale bars= 100  $\mu$ m.

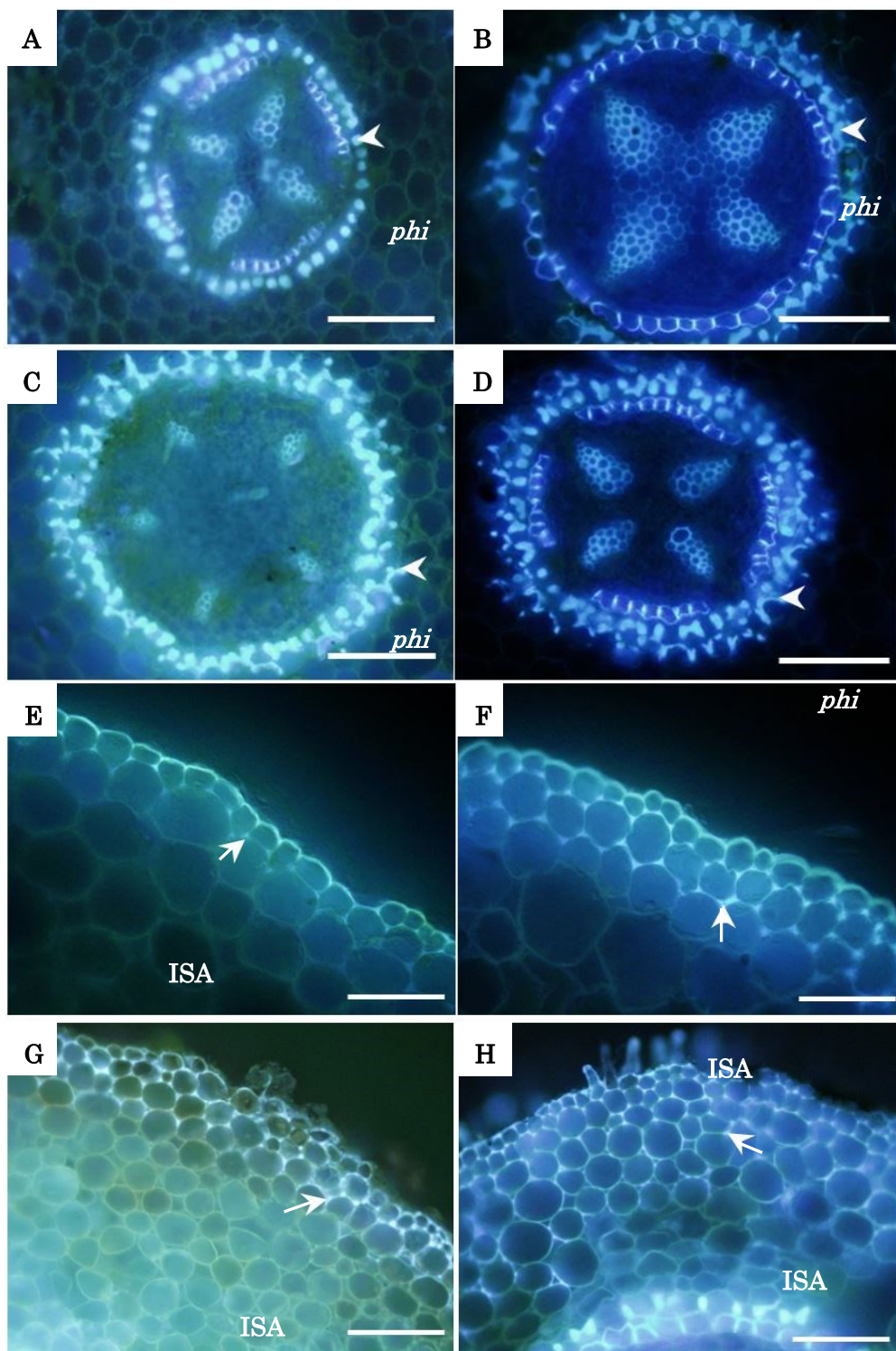


Fig.5-3: Extent of *phi*-thickening (*phi*) and intercellular suberin accumulation (ISA) classified into four grades in loquat roots for comparison among treatments. Panel A: grade 1, *phi* thickening appeared in the first cell layer of cortex adjacent to endodermis (+); panel B: grade 2, *phi* thickening appeared in the first to second layers (++); panel C: grade 3, *phi* thickening appeared in the first to third layers (+++); panel D: grade 4, *phi* thickening appeared in the first to four and more layers (++++); Arrowheads indicate *phi* thickening (A-D). Panel E: grade 1, intercellular suberin accumulation (ISA) appeared in the first cell layer under the epidermis (+); panel F: grade 2, ISA appeared in the first to second layers (++); panel G: grade 3, ISA appeared in the first to the third layers (+++); panel H: grade 4, ISA appeared in the first to fourth and more layers (++++). Arrows indicate ISA (E-H). Scale bars = 50  $\mu$ m (A- H).

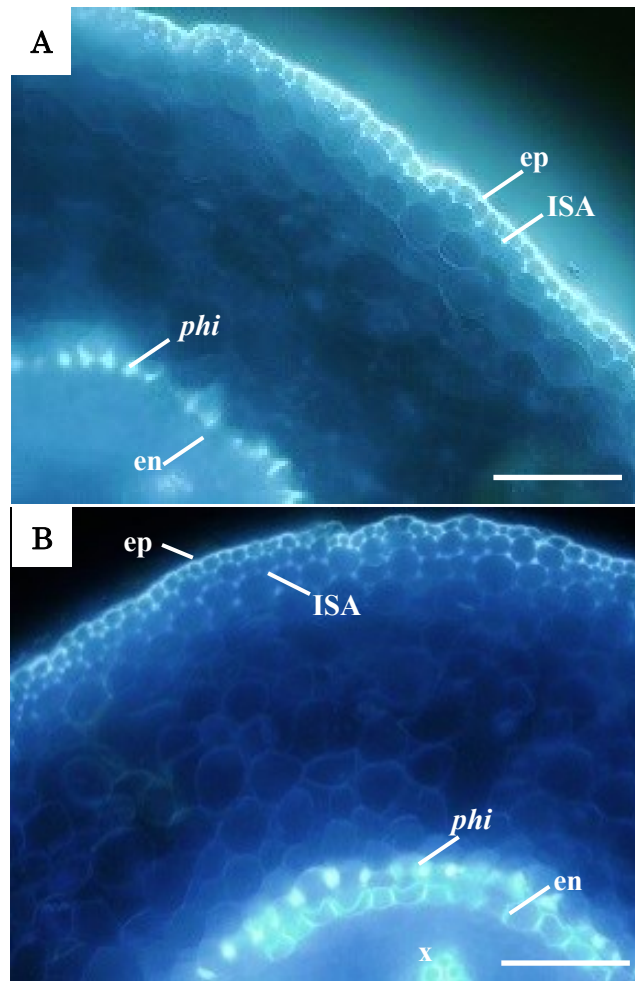


Fig.5-2: Existence of both *phi*-thickening and ISA before suberization of endodermis cells (panel A) and formation of Casparian strip at the endodermis (panel B) in cross-sections of root grown in normal conditions. Distance from the root tip panel A: 5 mm; panel B: 20 mm; en: endodermis with Casparian strip; ep: epidermis; en: endodermis; *phi*: *phi*-thickening, ISA: Intercellular suberin accumulation, x: xylem. Scale bars = 200  $\mu$ m.

## **General Summary**

Formation of such organizations as the International Society of Root Research (ISRR) and the Japanese Society of Root Research (JSRR) highlight the fact that root biology is gaining a lot of attention as a new area of research in plant science. Recently the 6<sup>th</sup> International symposium on physiological process in roots of woody plants was organized in Nagoya University, Japan 8-13 Sep. 2014. Study on woody tree root systems is an important focus area for scientists in the field of forestry. Fruit bearing trees have commercial importance in the field of agriculture. However, studies on anatomical features of fruit tree roots are scarce. In the present study, anatomical changes occurring in root structure due to soil conditions were studied in several fruit tree species. Hand sections and resin-embedded sections were observed after using several staining methods. Improved staining methods for hand section was employed, and root age was compared according to the development of xylem vessels instead of considering only the distance from the root tip.

The focus of the present study was on site-specific accumulation of lignin and suberin like biopolymers in the cell wall of root tissues such as epidermis, exodermis, endodermis, polyderm, and periderm. Although biochemical analysis of cell wall material was not conducted in this study, our results indicate that accumulation of such aliphatic and phenolic compounds in the cell wall of root tissues was influenced by soil conditions, root age and tree species.

The first three studies were dedicated to roots of Myrtaceae family, focusing on polyderm. Influence of soil conditions, root age and difference among tree species were studied. Result of the first study indicated that in Myrtaceae species, lignin-like accumulation was m-shaped in the epidermis but w-shaped in thick-walled cells of the

polyderm. It helped gain knowledge about site-specific accumulation of biopolymer according to root tissue. From the second study, it was known that the number of polyderm layers was fewer in plants grown in water-logged conditions than normal conditions and the third study highlighted results indicating earlier deposition of biopolymers in feijoa roots grown in drought conditions than normal or water-logged roots. From the fourth study, we came to know that unlike the Myrtaceae species, in Puniceae species, polyderm formation was absent. Alternative cell layers with lignin and suberin did not exist. Instead, both lignin and suberin existed in the same cell layer. This kind of periderm cell layers with brick-like accumulation of lignin seems to be a unique feature of Puniceae species. Finally, from the fifth study on loquat roots belonging to Rosaceae family, we know that cell wall ingrowth caused by lignin accumulation in cortex cells surrounding the endodermis called phi-thickening, decreased significantly in roots grown under water-logged conditions. Their presence was also confirmed in seed-generated roots and this was not confirmed before.

So far, anatomical studies depicting unique features of such a wide variety of fruit trees families have not been compared. In the present study, we have compared mainly three fruit tree families namely, Myrtaceae, Puniceae and Rosaceae. We have also attempted to examine several species of fruit trees belonging to the same family.

Further anatomical studies on biochemical accumulation in the cell wall in relation to disease and parasitic resistance and mycorrhizae could prove interesting to understand the adaptive response of plants for survival in challenging environment.

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