RESEARCH ARTICLE

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Anatomical observations on formation and development of adventitious root primordium in canes of *Vitis* sp.

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Abstract

Understanding the anatomical aspects of adventitious root primordium formation can provide valuable insights into the improvement of propagation techniques, rootstock selection, and overall vineyard management practices in *Vitis* spp. This work was carried out to investigate anatomical root primordium formation in the rooted cuttings of Cabernet Sauvignon (CS, *Vitis vinifera* L.) and the rootstock Kober 5BB (*Vitis berlandieri* x *V. riparia*) with their relationship to stem anatomy. One-node cuttings were grown under temperature-controlled conditions for 8 weeks. After removal of the roots and calli, the stem parts were fixed in a fixative solution. A revised method of safranin staining was applied to the 90 µm thick cross-sections made with a hand microtome. It was observed that root primordia were derived from the two different regions of the cane tissues: from the groups of cells close to the outside of the conductive tissue system and from the cell groups in the deeper site, close to the pith. Cultivars showed significant differences in terms of the regions where they had their root primordial initials. Number of potential root primordia was statistically higher in CS. Both CS and 5BB had root angles in the range of 83° to 86°. It was concluded that grapevine cuttings had only induced root primordia and the capacity to produce them was dependent on the genotype. Formation and development of root primordia and the anatomical differentiation of the cell groups were similar in Cabernet Sauvignon and 5BB.

Keywords: Cutting, Grapevine, Staining, Stem tissue

INTRODUCTION

Root formation is essential for vegetative propagation and plant growth. Root formation process varies by the genotype, and woody species are generally more difficult to root than herbaceous species (Hackett, 1988). Formation of root is still not fully understood, and it is not clear why cuttings of different cultivars have different rooting potentials. Information about anatomical events relating to root primordium formation is beneficial to improve our understanding rooting process in plants. The term of adventitious root (AR), by broad definition, refers to roots that arise from aerial plants parts, or underground stems (Hayward, 1983). In another sense, it refers to roots that do not arise from root pericycle tissue. The organization of adventitious roots structure and its development sequence is similar in all features to that of true roots. AR root primordia can be formed by stem cambium at specific sites where branch and leaf traces, parenchyma, or primary or secondary rays intersect with cambium tissue (Beakbane, 1961).

In grapevine growing, cuttings have been used to study root formation, but anatomical observations on initiation and development of root primordium generally are lacking. Van der Lek (1925) reported that adventitious roots of

Vitis vinifera after wounding were originated from the medullary rays. Subsequent anatomical studies on grapevine cuttings or canes were performed by Fujii (1955) and Han (1983) in which root primordia were reported to arise from outer part of vascular strands or from the cambium and the phloem ray, respectively. Furthering such studies would clarify developmental events leading to root formation.

Literature is abundant with the studies carried out on the rooting capacity of grapevine cuttings. However, studies on the events leading to formation of root primordia inside a grapevine cutting are not sufficient. The duration and location of these events can vary between taxa and even between conventional and micro propagated material (Lovell and White, 1986). Anatomic examination of root differentiation through staining techniques by taking thin sections from grapevine canes would reveal the regions where the root primordia are formed in the stem and contribute to a detailed understanding of the action mechanism of different applications on grapevine rooting.

Current methods of micro technique for microscopy that involve epoxy embedding and thin sectioning allow observation of subtle differences between cells less evident in cuttings. Today, new micro technical methods have been developed and taking thin sections (via microtomy) and staining techniques can enable observing events in cells especially in hard and woody species.

Understanding the anatomical aspects of adventitious root primordium formation can provide valuable insights into the physiological and molecular mechanisms underlying root development in *Vitis* spp. Furthermore, this knowledge can contribute to the improvement of propagation techniques, rootstock selection, and overall vineyard management practices. This research was carried out to examine origin sites of root primordia of *Vitis* fruit and rootstock cultivars and post-differentiation changes in the root primordia. Some quantification as to the frequency of occurrence of primordia at different sites was also done on the diameter and number of the root primordia.

MATERIALS AND METHODS

Stock Plants and Cuttings

Both Cabernet Sauvignon (CS, *Vitis vinifera*) and rootstock 5BB *(V. berlandieri* x *V. riparia*) are known for their ease in rooting. 2-node cuttings of 1 year-old CS and 5BB were planted in a rooting medium containing peat: perlite (2:1) after the basal node was removed and cut in the middle. The cuttings were grown in 1.9 L pots where their 2-3 cm basal parts were buried. Every pot had 10 cuttings for each treatment. The plants were maintained in a growth chamber at 24°C with a photoperiod of 16 h light (~ 900 µmol m^{-2} s^{-1} irradiance) and 8 h darkness. The mean diameter of the cuttings was ~1 cm. Following the period of 8 weeks, rooted stem parts were rid of any roots and callus as well as top growth. They were fixed in a fixation solution composed of glacial acetic acid, formaldehyde, and ethyl alcohol (FAA, 5: 10: 50) and stored in 100 ml dark brown bottles with low light transmission.

Microtechniques

For the purpose of visualization, cane parts were processed following protocols of Hacke (2015). Samples were removed from the FAA medium to section with a microtome, Before examination for root primordium development, the cross-sections were cleaned under a stereo microscope (Olympus SZ61, Olympus Corp., Japan) with the help of arrowhead needles and scalpel. During this process, the callus tissue and dead bark around the root exit site was removed without damaging the exit points in a solution containing ethanol and pure water (1:1) so that the tissues would not soften. There was no callus-root formation. Before taking the cross-sections, the surface of the samples was dried and tightly wrapped with an adhesive resin tape close to the top to prevent the tissues from disintegrating. For each stem part, 5-6 mm internode fragments were sectioned.

Taking cross-sections as thin as possible is of great importance for successful microscopic examinations. For this purpose, a classical sliding semi-automatic microtome (Reichert Jung, Germany) was used. The thickness of the cross-sections taken was 90-100 µm on average, and very sharp blades were used and changed frequently. After adjusting the microtome blade to a section thickness about 100 µm, a drop of 98% ethanol was placed on the surface of the sample, and the excess on the sample was removed with the help of a wet watercolor brush. This process prevented the blade contact point from getting wet and bending the cross section. The cross-sections were later put into petri dishes containing 98% ethanol before proceeding to the staining procedure. The cross-sections taken were examined under the microscope (Olympus SZ61) and checked for traces and cracks that might result from the cutting edge of the microtome blade, and such samples were not included in the staining test.

Staining of the cross-sections were carried out by the modified methods of different researchers (Bond et al., 2008; Hacke, 2015). Sections were washed three times with distilled water for 10 minutes followed by immersion in 1g/L safranin solution for 20 minutes. Later they were rinsed three times in distilled water to remove excess stain. Then they were placed in 0.75% bromophenol blue solution (including 10% glycerol and 10% acetic acid) for 25 minutes and again rinsed three times in distilled water.

Microscopy

Observations of stem tissues and root primordia were carried out under stereo zoom microscope (Olympus SZX7, Olympus Corp., Japan) equipped with a digital

camera (Olympus LC20, Olympus Corp., Japan) and, when permitted, under the light microscope (Olympus CX41, Olympus Corp., Japan). The LCmicro software (Tokyo, Japan) was used for measurements and counting. Cross-sectional diameter of root primordium was calculated on each cross-sections considering at last 5 primordia, then from a total of 75 primordia for each cultivar. Sites for the root primordia were also determined. Root exit angle was determined by measuring the angle with respect to the horizontal line against the slope of the root exiting the epidermis.

Statistical Analysis

Values represent the means of three replicates per cultivar. Data for diameter of root primordium and for the other parameters were collected from n=75 and 45, respectively. They were evaluated by ANOVA (analysis of variance) and comparisons between the mean values were made according to the Tukey's t-test at $p< 0.05$.

RESULTS AND DISCUSSION

Formation and development of root primordia and the anatomical differentiation of the cell groups were similar in Cabernet Sauvignon and 5BB. It has been observed that many cells in the canes of CS and 5BB had the ability to go back to meristematic status and form roots.

Statistical analysis showed that genotype played a major role in the characteristics belonging to AR primordia formation in the stem tissues (Table 1). It was observed that both cultivars had quite a high number of dense cell groups that might be considered as potential primordia. In terms of the site for these primordia, CS had considerably more root primordia, most of which originated near the vascular elements. On the other hand, 5 BB produced almost twice the number of root initials in its deeper region compared to its vascular system. Root diameters in CS were comparably thinner than those in 5BB. Root angle was similar in both genotypes. Root angle was quantified as the average 83° in 5BB and 86° in CS.

Cell groups divided to form many new cell groups and these groups turned into root primordium (Fig. 1). In each cell group, division continued (Fig. 1a) and took the appearance of root primordia initials (Fig. 1b). Once initiated, it was observed that root primordium (Fig. 1 b, c) enlarged until it reached the outside of the tissue system and emerged towards the outer bark surface of the cane (Fig. 1d).

It was determined that the root primordia were generally stemmed from two different regions of the tissues (Table 1, Figure 2). Although the roots originated from the cells in files outside and between the conductive tissues, connections of cell groups extending to the pith were also observed in some of the samples. Roots which arose within the phloem or vascular cambium in both 5BB (Figure 2a) and CS (Figure 2b) indicated a direct AR formation that did not involve callus formation. The roots grew through the cortex and penetrated the epidermis (Figure 2c, d). Figure 2e shows a more highly magnified view of the xylem and phloem elements in a cleared root of CS and figure 2f shows view of the xylem elements of the two cleared root of 5BB.

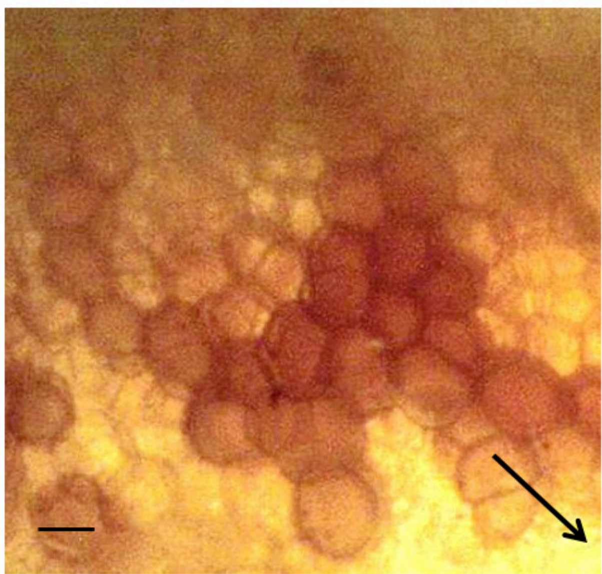
Another group of cells in the deeper regions extending to the pith were also observed to be competent to form AR initials (Figure 3). These cell groups were divided to form other cell files, and these cell files turned into root primordia. Some root primordia could also be observed between the pith and xylem or rays (Figure 3b). New root primordia continued to develop and connected to the nearest conductive tissue system (Figure 3c, d). Figure 3e shows a more highly magnified view of the new root with cambial continuity initiated from pith and xylem elements in 5BB cross section. The new root was grown outward through the cortex and penetrated the epidermis forming an angle of 83-86° with the cane (Figure 3f).

The anatomical observations in this research have confirmed that *Vitis* species do not have pre-formed root primordia in their canes, as also stated by Smart et al. (2003). They have what is called an induced primordium, which is formed upon a stimulatory effect as wounding by cutting or application of growth regulators (Tailor et al. 2022). Initiation of the primordium is usually

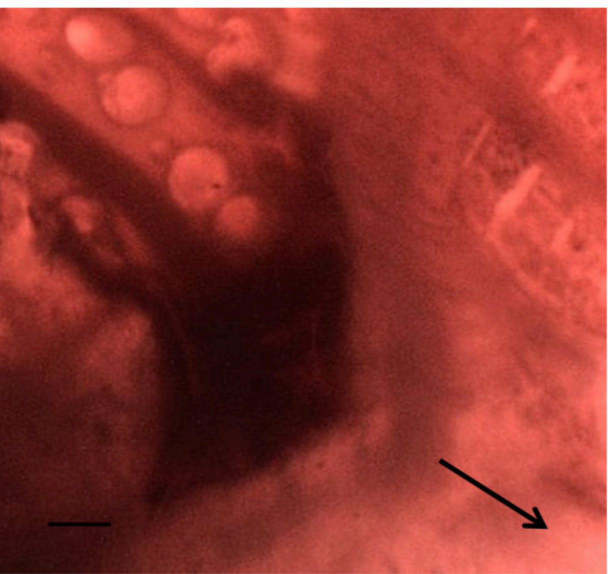
Table 1. Measurements of root primordia number, root diameter, root angle and number of root primordia in 5BB and CS

	Cultivars	
	5BB	**Cabernet Sauvignon**
Total number of potential root primordia (n)	11,83 b*	25,37 a
Number of root primordia close to the outside of the conductive system (n)	4,300 b	16,73 a
Number of root primordia from deep and pith region (n)	7,53 b	8,63 a
Root diameter (µm)	353,70 a	153,30 b
Root angle (°)	83,47 a	86,41 a

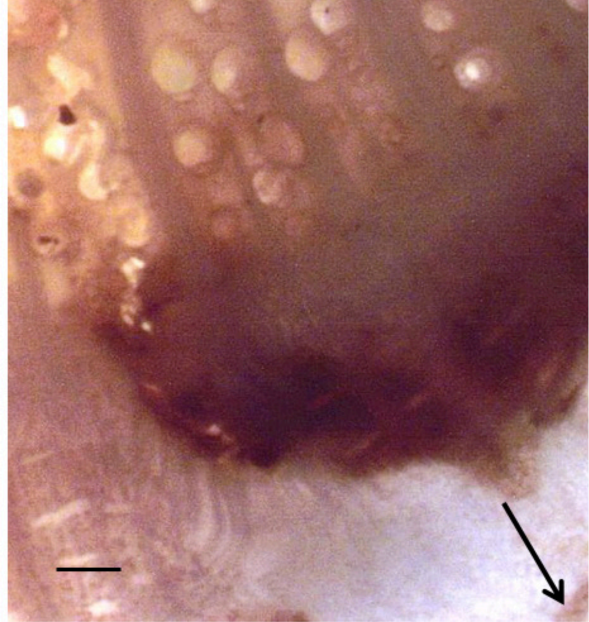
*Means in each column followed by the same letters are not significantly different at p <0.05 according to Tukey's t-test.



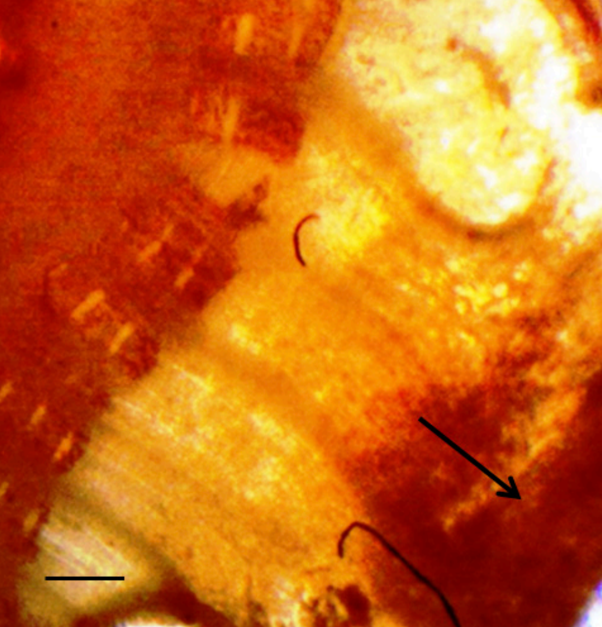
a) A site for primordium initiated. Pericycle cells going through radial expansion and one tangential division.



b) Each cell continues to expand radially, forming an arc. Outer cells continue radial enlargement and tangential division. Protoplasmic content increases in some of the endodermal cells. Radial cell lines were formed by tangential divisions of both the outer and inner rows of pericycle cells.

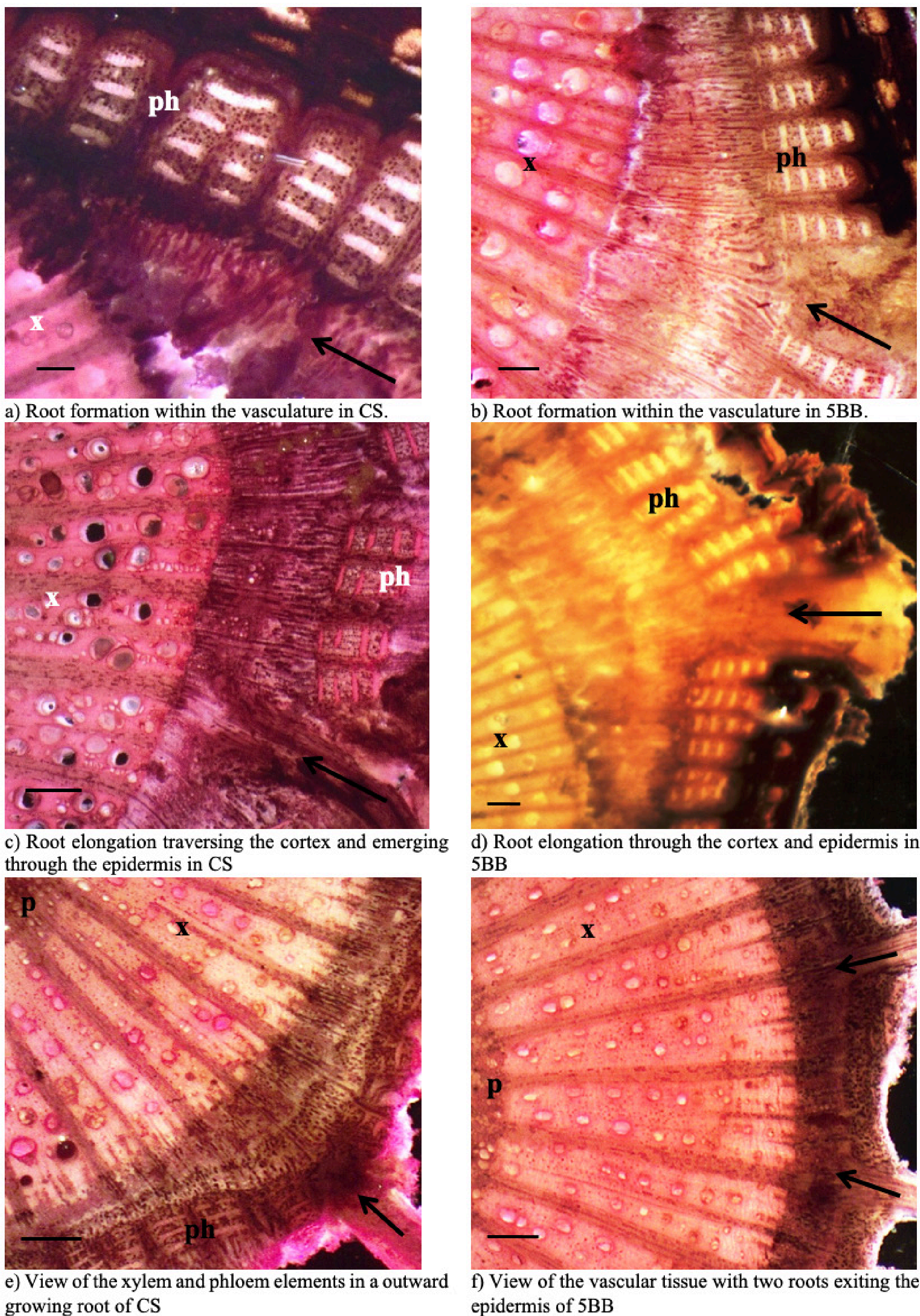


c) Cells of radial growth by the root primordium. The root primordia is almost traversing the cortex.



d) A root primordium just completing penetration through the cortex and epidermis.

Figure 1. Root primordium formation and development in 5 BB. The arrow shows the direction of penetration of a root primordium. Bar=100 µm.



a) Root formation within the vasculature in CS.

b) Root formation within the vasculature in 5BB.

c) Root elongation traversing the cortex and emerging through the epidermis in CS

d) Root elongation through the cortex and epidermis in 5BB

e) View of the xylem and phloem elements in a outward growing root of CS

f) View of the vascular tissue with two roots exiting the epidermis of 5BB

Figure 2. Root formation site and roots elongating through the cortex and emerging out of the epidermis in 5BB and CS cross sections. Arrows indicate root primordia in the vascular tissue. ph = phloem, x = xylem, p = pith, Bar=100 µm.

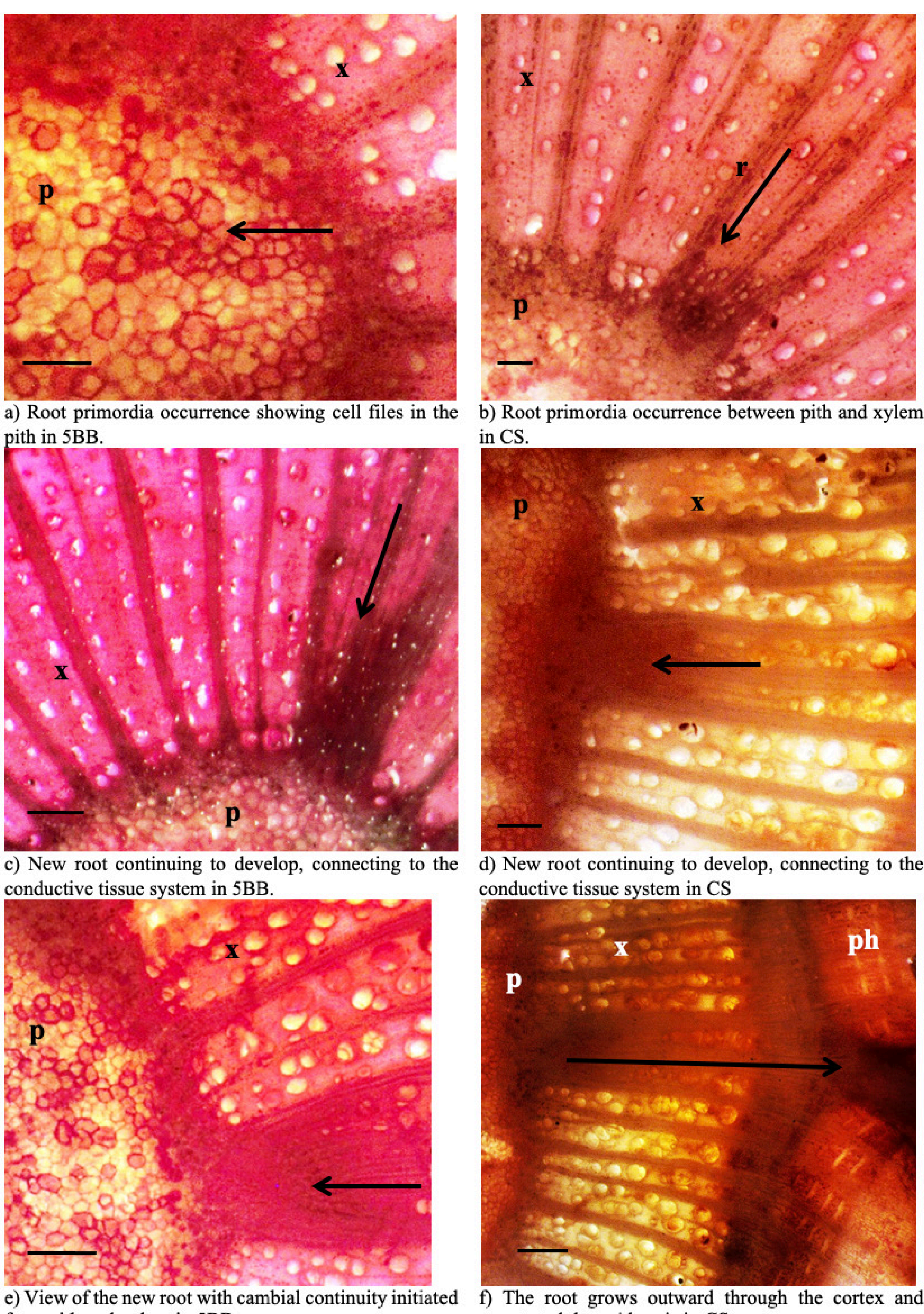


Figure 3. Root formation site and the new root primordium with cambial continuity initiated from deep and the pith, and roots passing through the cortex and epidermis in 5BB and CS cross sections. Arrows indicate new root development in cane tissues. ph = phloem, x = xylem, p = pith, r = ray, Bar=100 µm.

accounted for the dense cell groups with large nuclei and small vacuoles (Cameron and Thomson, 1969) and in this study *vinifera* had considerably had more compared to the *Vitis* hybrid. However, it does not mean that all of these cell lines would be determined to be a developing root (Lovell and White 1986). This is the case here too in which the number of the committed initial primordia was less than root primordia.

Once the root primordia initiated, it grows and enlarges until it reaches the outer bark surface. Root primordia could be formed by stem cambia at specific sites where branch and leaf traces, parenchyma, or primary or secondary rays that intersect with cambium tissue (Roy et al., 1987). Our results show that rooting is not restricted to the cambium zone and may develop at pith and cortex or any point along the vascular tissues and through xylem if there is retention of meristematic capacity in certain cells. In both cultivars, initiation of cell division for root formation took place in parenchyma cells of the phloem, cortex, cambial region, xylem, and pith. In studies of propagated other species, cells leading to root formation could have been in cells within or just external to the vascular cambium (Vieitez and Vieitez, 1983; Samartin et al., 1986; Hicks, 1987; Ranjit et al., 1988; Isfendiyaroğlu and Ozeker, 2008). Formation of root primordium occurred in the same way in both cultivars and no significant difference was determined. *Vitis vinifera* cuttings have been accepted as easy to root, compared to other *Vitis* hybrids and higher number of potential root primordia and them being located near epidermis in conjunction with vasculature would certainly support this notion. In addition to this, reactions of root formation are probably related with the presence or content of endogenous auxin in such cuttings (Koll et al., 2012).

Root diameter is considered one of the characteristics used for determination of root system architecture (Dumont et al. 2016). The rootstock 5BB is known for abundant propagule production due to its profuse rooting. Considerable difference in root diameter between 5 BB and Cabernet Sauvignon show genetical variations. The enlargement of the root diameter is associated with the inhibition of cortical cell division (Koll et al., 2012). The reason why the 5BB had thicker roots is perhaps due to higher levels of endogenous auxin concentrations and subsequent changes in cell number and size of the cortical area reflected in the root diameter.

Root angle is another factor for root system architecture, determining whether a plant develops deep or shallow roots, as it determines the direction of root elongation (Kitomi et al., 2015; Uga et al., 2015). Previous work with root angle states that roots in the two growth angle categories 0° to 45° and 45° to 90° from the horizontal line (Ramalingam et al., 2017). Many studies have examined the relationship between root angle and development in other crop species (Kato et al., 2006; Ali et al., 2015; Dathe et al., 2016). However, until now, root angle analysis studies on grapevines have not been done sufficiently. In the current study, both Cabernet Sauvignon and 5BB had root angles in the range of 83° to 86° and the difference was important, although in their study Schmitz et al. (2021) reported genotype differences in adventitious root angle between three grape cultivars. Another observation in this study was that the roots forming from deep sites and pith were at an angle close to 90° and that those from outside of the conductive system were at an angle of 70°-80°, but this needs confirmation with further studies.

CONCLUSION

Rooting ability in woody trees highly depends on their ability to turn a group of cells to dedifferentiate into meristematic form and then, develop into root primordia initials. Following sequential events that lead to adventitious root formation in the stems have been lacking in grapevines since taking and staining thin slices have proved to be not as easy and successful as that in herbaceous plants. However, successful efforts have widened our view on formation and development of root primordia and increase our understanding of the effects of wounding after cutting, growth regulators, and stress factors. This study indicated that *Vitis* species have induced primordia and the site they derive is genotype dependent. Further anatomical studies in which time-course changes from planting a cutting to uprooting are observed would allow, especially in hard-to-root species, to sequence adventitious root initiation and development inside stems.

COMPLIANCE WITH ETHICAL STANDARDS

Peer-review

Externally peer-reviewed.

Declaration of interests

The authors declare that they have no competing, actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethics Committee Approval

Ethics committee approval is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

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Data availability

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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