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EFFECT OF CUTTING MEDIUM TEMPERATURES ON ROOTING PROCESS AND ROOT PRIMORDIUM DIFFERENTIATION OF HARDWOOD CUTTINGS OF TETRAPLOID *ROBINIA PSEUDOACACIA* CUTTING MEDIUM TEMPERATURES OF TETRAPLOID *ROBINIA PSEUDOACACIA*

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Abstract

In this study, to examine the effect of heat treatment on the rooting and root development of hardwood cuttings of the tetraploid *Robinia pseudoacacia*, cuttings of 1-year-old stems were taken from 3–year-old mother trees and treated with IBA solution (1000 mg/L) for 6 h, with water was as a control. Treated cuttings were rooted in heated or unheated nursery beds. Samples were collected on day ten after planting, and then for every five days. The bases of the cuttings were embedded in paraffin and sectioned before being examined under a microscope to determine whether there had been any morphological changes. We found no root primordia in the tissues of the hardwood cuttings of the tetraploid *Robinia pseudoacacia* before cutting. In the heated bed, adventitious roots originated from callus tissue and the junction between the pith rays and cortical parenchyma cells, and in the unheated bed, adventitious roots originated only from callus tissue. The rooting process involved callus formation, adventitious root formation and elongation; rooting occurred 5-7 days earlier in the heated cuttings than in the unheated ones, and rooting rates were significantly higher in the former 30 days and 50 days after cutting; the minimum effective accumulated temperatures for these three stages were 109.25°C, 211.68 °C and 301.38°C, respectively. Our results revealed that heating the soil can promote adventitious root formation, speed up the rooting rate, and cut the propagation period of the tetraploid *Robinia pseudoacacia*.

Key words: Primordium differentiation, Hardwood cuttings, Tetraploid *Robinia pseudoacacia*.

Introduction

The tetraploid *Robinia pseudoacacia*, generated by artificially inducing chromosome doubling in the diploid species cells, has several desirable traits. They include strong tolerance of low temperatures, drought, nutrient deficiency, dust and salinity. The tetraploid *Robinia pseudoacacia* is considered one of the best tree species for maintaining natural environments in western China (Wang *et al*., 2011). In recent years, the demand for some excellent cultivars has been increasing. Hence, the propagation method is worth special attention.

Tetraploid *Robinia pseudoacacia* plants are propagated mainly by grafting. The major shortcoming of this method is that the plants take longer to mature. It is necessary to culture as a rootstock for 1-2 years before grafting (Wang & Zhao, 2012). Then, it can be transplanted 2-3 years later. Although grafting cultivars can successfully establish a new tetraploid *Robinia pseudoacacia* orchard, the low productivity ability and unsatisfactory viability in some cultivars were identified as limiting factors (Sebastiani & Tognetti, 2004).

Propagation by cuttings generally has high propagation coefficients and can be applied to large-scale plantations. This is why the method is widely used for asexual propagation of many types of trees. However, tetraploid *Robinia pseudoacacia* cuttings are difficult to root (Swamy *et al*., 2002a, 2002b), and its hardwood cuttings are more difficult to root than its softwood cuttings (Kasim *et al*., 2009). Many studies show that rooting response correlates with the interactions between endogenous plant hormones (Tsipouridis *et al*., 2006). Auxin is believed to play a central role in the formation of adventitious root (Hussain *et al.,* 2013). We also found that higher contents of indole-3-acetic acid can inhibit the rooting of tetraploid *Robinia pseudoacacia* hardwood cuttings. Little is known about the mechanisms underlying adventitious root formation, indicating the necessity for further study.

Therefore, the goals of this work were (a) to elucidate the morphological and anatomical changes during the rooting process and (b) to quantify the effects on the rooting rate and rooting process in hardwood cuttings of the tetraploid *Robinia pseudoacacia* with heat treatment (heated and unheated).

Materials and Methods

Trial sites: The experiment was conducted in an experimental nursery of the Forestry College, Northwest Agriculture and Forestry University (108°07'E, 34°12'N), Yangling, Shaanxi; the area has a semi-moist warm temperate climate. The altitudinal range at the site is 403.2-530.1 m, with an average annual accumulated temperature of 4811°C, an average annual temperature of 12.9°C, and minimum and maximum temperatures of -19.4°C and 42°C, respectively. The average annual precipitation is 660 mm and the annual frost-free period is more than 220 d, with annual averages of 2163.8 h of sunshine and 114.8 KJ/m^2 of total radiation.

Materials: One-year old hardwood shoots (diameter 0.8-1.2 cm) of three-year old tetraploid *Robinia pseudoacacia* plants grafted onto rootstocks from diploid seedlings were excised on February 27, 2010, and cuttings (12-15 cm long) were obtained from the central 50 cm sections of the shoots. The upper end of the cutting was cut at right angles to the stem and the bottom was cut obliquely, approximately 1 cm below an axillary bud.

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Nursery beds: Two nursery beds (12 m×8.5 m) were built in a south-facing position. A layer of pebbles (15-20 cm deep) was placed on the bottom of each bed, followed by a layer of sawdust (approximately 8 cm deep); then 800 w electric heating wires were laid across one of the beds to be used for the heat treatment. A layer of sand (15 cm in depth) was laid over the wires in the heated bed or directly onto the sawdust in the unheated bed. The nursery beds were divided into three areas from east to west. Buffer zones 0.5 m wide were established between the adjacent areas. Three days before the cuttings were introduced, the beds were sprayed with carbendazim to disinfect them.

Experimental treatments: Heat treatments: over the experimental period (March 1, 2010 to April 20, 2010) a temperature of 26±0.5^{o}C at a depth of 8 cm from the surface was maintained in the heated bed, whilst the other bed remained unheated; the actual temperatures in the two beds were recorded hourly using a TNHY-4 temperature logger. The effective accumulated temperature (≥10^{o}C) was calculated.

Cuttings: Bundles of 50 cuttings were first soaked in 5 g/L carbendazim solution for 3 min and then air dried. Based on our pre-test data, cuttings were then soaked in a 1000 g/L IBA solution. The 5 cm ends of the cuttings were immersed in the IBA solution for 6 h, and water was used as a control. A randomised block design was employed for the experiment: 300 cuttings were exposed to each of the heat treatments, with 100 cuttings per block. The cuttings were inserted to a depth of 8 cm in the sand beds, at a density of 400 cuttings/m^2.

Morphological and anatomical observation of the rooting process: We began sampling ten days after the cuttings were planted in the beds; samples were then collected and examined every 5 d, until the roots emerged. On each sampling occasion, six cuttings were randomly selected from each treatment. Morphological changes in the bottom 1 cm of the cutting were recorded, as well as callus size and colour. Time of root emergence was also noted. The cuttings were sectioned 0.5 cm above the base, fixed with FAA for 24 h, and softened in softening solution (50% ethanol in glycerol) for 15 d. The specimens were then embedded in paraffin and 10 μm thick sections were cut. The sections were stained using Safranin /Fast Green dye, cleared in xylene and sealed with neutral resin. Observations were conducted using an OLYMPUS (BX41-32P02) universal microscope.

Rooting rates: The rooting rate 30 days after cutting was determined on the basis of 40 cuttings selected randomly from each treatment. At the end of the experiment (50 days after cutting), final rooting rates were determined based on all remaining cuttings.

Statistics: The data were evaluated by Analysis of Variance (ANOVA) using SPSS 16.0 for Windows software. Rooting rates were arcsine-transformed so that they were normally distributed. The significance of differences between the treatment-related mean values was assessed using the Least Significant Difference (LSD) test.

Results

Morphological changes in the cutting bases: Approximately 10 d after planting in the heated bed, a small amount of white callus had appeared on the wounded surfaces of the cuttings (Fig. 1-a). Over the period of 10-15 d after the cuttings were taken, protruding callus tissues appeared, forming a circle around the wound and turning yellowish. Meanwhile, the upper buds of the cuttings began to sprout (Fig. 1-b). During the period of 15-20 d after the cuttings were taken, the callus tissues turned brown, and a large number of leaves were produced by the cuttings, which started to expand. At this stage, callus tissues were well developed in the heated cuttings (Fig. 1-c). During the period of 20-25 d after the cuttings were taken, the callus turned from yellow to brown, and tiny adventitious roots, which were visible as white spots, formed on the brown callus produced earlier (Fig. 1-d). Over the period of 25-50 d after the cuttings were taken, a large number of adventitious roots emerged and developed (Fig. 1-e).

On the 15th day after the cuttings were taken, a small amount of white callus appeared on the unheated cutting bases, five days later than was the case for the heated cuttings (Fig. 2-a). Over the period 15-25 d after the cuttings were taken, the callus on the wound sites of the unheated cuttings gradual turned yellow (Fig. 2-b). During the next five days, the yellow callus gradually turned brown. After this stage, the callus was morphologically stable, 10 days later than was the case for the heated cuttings (Fig. 2-c). Over the period of 30-35 d after the cuttings were taken, white and translucent adventitious roots were observed on the brown callus tissues (Fig. 2-d). Some adventitious roots emerged above the wound sites 35 d after cutting (Fig. 2-e).

Anatomical changes in the cutting base during the rooting process: Anatomical observations revealed that temperature affected the morphogenesis of adventitious roots of the tetraploid *Robinia pseudoacacia*. In the heated cuttings, the adventitious roots differentiated from the callus and from the parenchyma cells at the junction between the pith rays and the cortex. In the unheated cuttings, the adventitious roots differentiated only from the callus tissue.

Differentiation of the adventitious roots from the parenchyma cells could be divided into three stages. In the first stage, the parenchyma cells at the junction of the pith rays and cortex produced calluses (Fig. 3-a). The parenchyma cells then continued to differentiate to form root primordia. At the junction of the pith rays and the cortex, there was a group of cells that exhibited high division efficiency; this group was small, dense, deeply stained, and quite different from the surrounding cortex parenchyma cells. These cells developed into root primordia (Fig. 3-b). In the last stage, adventitious root formation and elongation were observed (Fig. 3-c). Formation and development of the callus and root primordia occurred almost simultaneously. On the 35th day, the adventitious roots could be observed protruding from the cuttings.

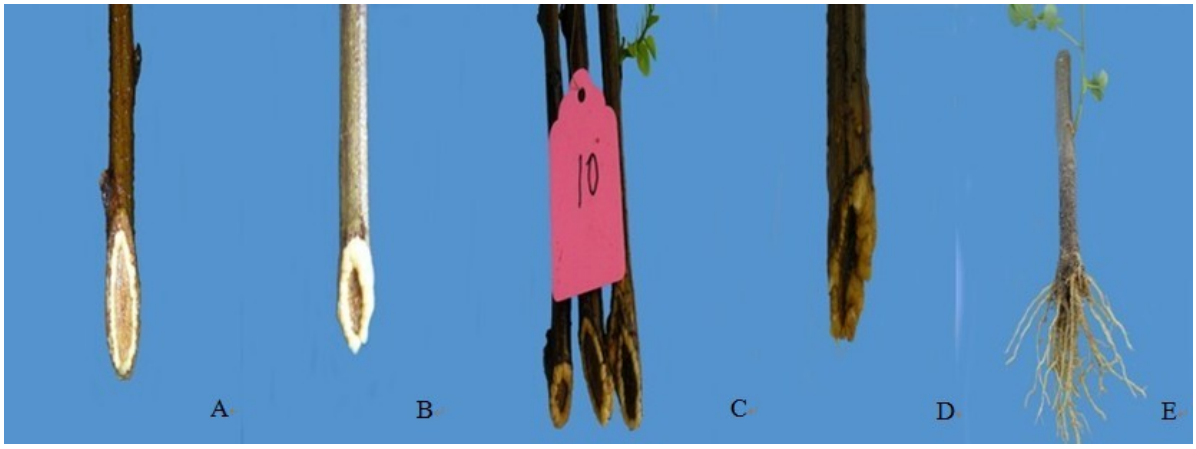

Fig. 1. Morphological changes in tetraploid *Robinia pseudoacacia* cuttings undergoing adventitious root development in the heated bed. (a), white callus appeared 10 d after cutting. (b), yellow callus appeared 15 d after cutting. (c), brown callus appeared 20 d after cutting. (d), tiny adventitious roots emerged 25 d after cutting. (e), adventitious roots 50 d after cutting.

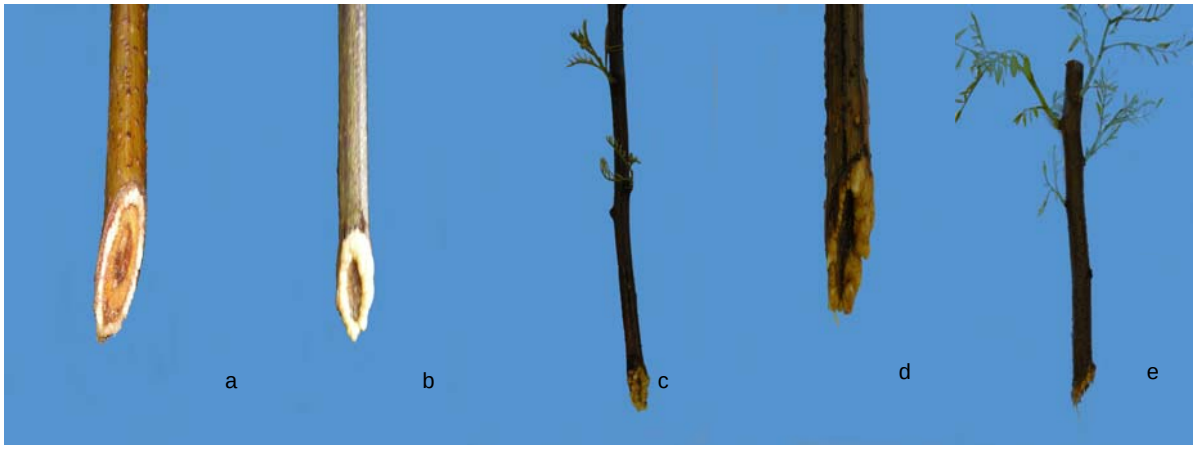

Fig. 2. Morphological changes in tetraploid *Robinia pseudoacacia* cuttings undergoing adventitious root development in the unheated bed. (a), white callus appeared approximately 15 d after cutting. (b), yellow callus appeared approximately 25 d after cutting. (c), brown callus appeared approximately 30 d after cutting. (d), tiny adventitious roots appeared approximately 35 d after cutting. (e), adventitious roots emerged 35 d after cutting.

Differentiation of the adventitious roots from the callus could also be divided into three stages. In the first stage, formation and development of the callus occurred (Fig. 3-d; Fig. 4-a). The initial callus comprised a group of parenchyma cells, which were large, nearly round, loosely arranged, and in which the nuclei were unclear. In the second stage, calluses that differentiated during the first stage developed root primordia (Fig. 3-e, Fig. 3-f, Fig. 3-g, Fig. 3-h, Fig. 4-b, Fig. 4-c, Fig. 4-d, Fig. 4-e). As the number of callus cells increased, the cells inside the callus tissue began to differentiate and those with thicker cell walls developed into primitive root cells. Some cells became specialised, exhibiting features of primitive root cells, distinct from the surrounding callus cells. From these specialised cells, tracheary elements were produced, as well as root primordia; the cambium layer also formed in the callus tissue. In the third stage, formation and elongation of adventitious roots occurred (Fig. 3-i, Fig. 3-j, Fig. 4-f). As root primordia formed at numerous sites and at different times in the callus tissues, many adventitious roots emerged over a number of days.

Effect of temperature on the rooting rate: Adventitious roots emerged from the heated cuttings after 30 days, five days earlier than from the unheated ones (in Table 1). On the 50th day after cutting, rooting rates for the heated cuttings with and without IBA treatment were 80.7% and 50%, respectively. When untreated with IBA, the unheated cuttings failed to root. Those treated with IBA had a rooting rate of 8.64%.

ANOVA indicated that on the 30th day after cutting, the rooting rates for the different treatments were significantly different. On the 50th day after cutting, the rooting rates for the heated cuttings treated with IBA and the water control were also significantly different, but in the unheated bed there was no significant difference in rooting rate between the IBA treated and control cuttings. Our data indicated that temperature had a significant effect on the rooting rate of cuttings of the tetraploid *Robinia pseudoacacia* and played a key role in the rooting process.

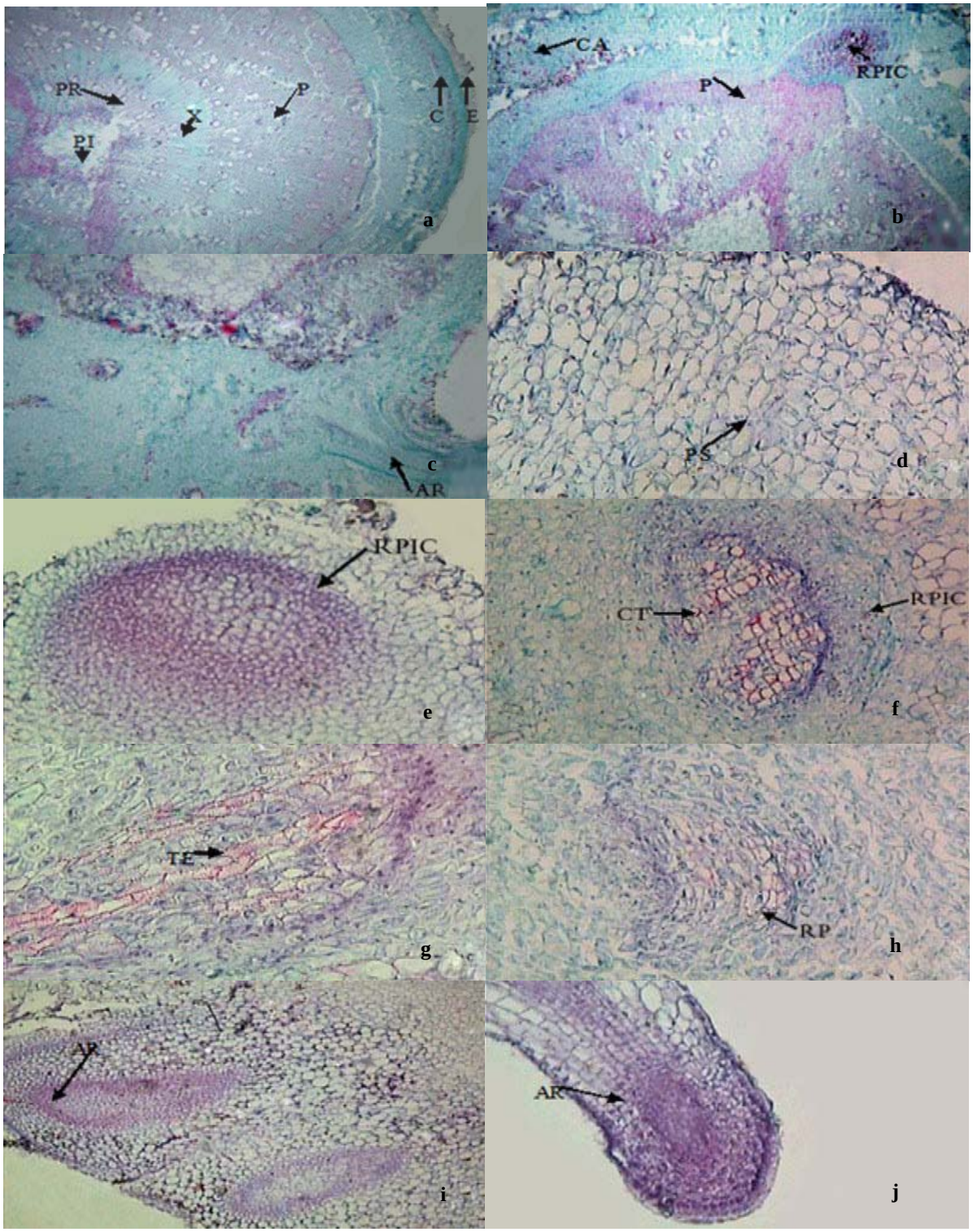

Fig. 3. Anatomical changes in tetraploid *Robinia pseudoacacia* cuttings undergoing adventitious root development in the heated bed, E-periderm, C-corium, P-phloem, X-xylem, PI-pith, PR-medullary ray, VC- vascular cambium, CA-callus, RPIC-root primordial cell, RP-Root primordial, AR-adventitious roots, CT-callus conducting tissue, PC-parenchyma cells. (a) cross section of the stem before cutting (20×). (b) root primordium differentiated from callus and the parenchyma cells at the junction between the pith rays and cortex 25 days after cutting (40×). (c) Adventitious roots appearing 30 days after cutting (40×). (d) Parenchyma cells of callus 15 days after cutting (40×). (e) Root primordial cells differentiated from callus 25 days after cutting (40×). (f) Drive pipe differentiated from root primordial cell of callus 30 days after cutting (40×). (g) Tracheary elements of conductive tissues appeared 30 days after cutting (40×). (h) Root primordia induced from callus 30 days after cutting (100×). (i-j) Adventitious roots protruding from callus 35 days after cutting (100×).

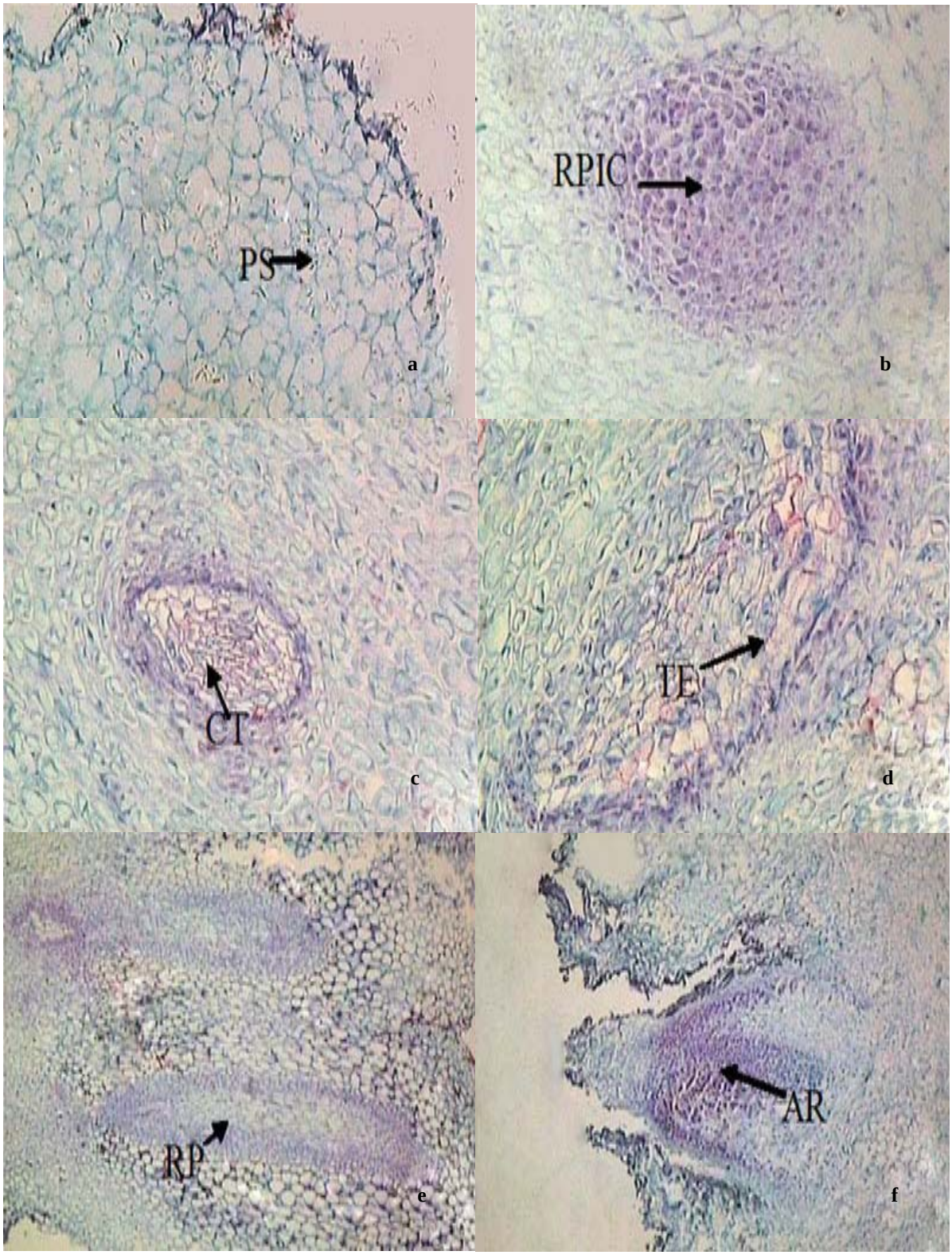

Fig. 4. Anatomical changes in tetraploid *Robinia pseudoacacia* cuttings undergoing adventitious root development in the unheated bed, CA-callus, RPIC-root primordial cell, RP-root primodia, AR- adventitious roots, CT-callus conducting tissue, PC-parenchyma cells. (a) parenchyma cells of callus 20 days after cutting (40×). (b) root primordial cells differentiated from callus 25 days after cutting (40×). (c) drive pipe differentiated from root primordial cell of callus 25 days after cutting (40×). (d) tracheary elements of conductive tissues appeared 25 days after cutting (40×). (e) root primordia induced from callus 30 days after cutting (40×). (f) adventitious roots protruding from callus 40 days after cutting (100×).

Table 1. Rooting rate of tetraploid *Robinia pseudoacacia* cuttings with and without heat.

Heat treatment	IBA treatment (mg/L)	Initial rooting rate		Final rooting rate	
		Days after cutting (d)	Rooting rate (%)	Days after cutting (d)	Rooting rate (%)
Without heat	0	35	0D	50	0C
Without heat	1000	35	5 ± 0.0C	50	8.6 ± 1.7C
With heat	0	30	10 ± 1.4B	50	50.0 ± 2.0B
With heat	1000	30	35 ± 1.4A	50	80.7 ± 7.0A

For each parameter, means within columns with different letters are significantly different at the $p<0.01$ probability level according to the LSD test

Table 2. Data on root development for combinations of the treatments with/without heat and with/without IBA for tetraploid *Robinia pseudoacacia*.

Heat treatment	IBA treatment (mg/L)	Callus formation stage		Root primordia differentiation stage		Adventitious root elongation stage	
		Days after cutting (d)	Effective accumulated temperature (^oC)	Days after cutting (d)	Effective accumulated temperature (^oC)	Days after cutting (d)	Effective accumulated temperature (^oC)
With heat	0	15	194.17	25	359.01	30	459.49
With heat	1000	15	194.17	25	359.01	30	459.49
Without heat	0	20	109.25	30	211.68	37	301.38
Without heat	1000	20	109.25	30	211.68	37	301.38

Effect of temperature on the rooting process: The effective accumulated temperature could be the trigger for the promotion of calluses, root primordia and adventitious roots (in Table 2). Under the heat treatment, the effective accumulated temperatures for the formation of the calluses, root primordia and adventitious roots were 194.17^{o}C, 359.01^{o}C and 459.49^{o}C, respectively; these temperatures are 84.83^{o}C, 148.33^{o}C and 158.11^{o}C higher than their counterparts in the unheated treatment. Root formation by the heated cuttings occurred 5-7 d earlier than in the unheated cuttings.

Our data indicated that cuttings of the tetraploid *Robinia pseudoacacia* produced calluses, formed roots and commenced root elongation only when the effective accumulated temperature in the growing medium exceeded 109.25^{o}C, 211.68^{o}C and 301.38^{o}C, respectively. IBA treatment may affect the rooting rate, but it has no effect on the timing of the different stages.

Discussion

Adventitious rooting formation in stem cuttings is influenced by diverse internal and external factors (Gyand, 2006). For example, auxin applications can increase the rooting capacity of many plants (Kiran & Kaul, 2008). Therefore, auxin (generally IBA) is widely used in commercial propagation (Kotis *et al*., 2009). This study showed that IBA treatment (1000 mg/L) increased rooting rate of the tetraploid *Robinia pseudoacacia* compared to plants not treated with IBA. IBA enters the hardwood cuttings' tissue and can quickly begin amino acid binding. Then, they become inactive bound auxin. In addition, IBA can be transformed into IAA, and IAA can promote rooting (Hussain *et al.,* 2011). Finally, IBA can promote cell division and the elongation of the cuttings and strengthen starch and fat hydrolysis, which are beneficial for nutrient flow to the base of the cuttings. Thus, IBA can promote rooting.

Zhang *et al*., (2009) suggests that adventitious roots originate from root primordia, which can be divided into two types, latent and induced, according to their formation time. The former is the dormant type, present in stems before cutting and developing into adventitious roots under suitable environmental conditions. The induced root primordia are formed only after cutting as a result of induction and subsequently develop into adventitious roots. The latter do not exist before cutting. Anatomical observation revealed that there were no root primordia in the tetraploid hardwood cuttings; all root primordia are formed as a result of IBA induction (Blakely *et al*., 1988).

Cuttings of different plant species have different adventitious root initiation sites (Fatma & Balta, 2004): they can be produced from the bark layer, callus or both (Hubl *et al*., 1984). All species with cuttings that are hard to root most likely belong to the group in which adventitious roots are generated from callus tissues. For example, in the cuttings of *Ulmus pumila*, *Populus* spp., *Cryptomeria fortunei*, *Sophora japonica*, *Taxodium distichum* and *Taxus cuspidata*, adventitious roots are produced from callus tissues at a wound site, and callus formation is a prerequisite for rooting (Hussain *et al.,* 2013). Our observations suggest that adventitious roots of the tetraploid *Robinia pseudoacacia* largely originate from calluses.

Successful cutting propagation is also associated with appropriate external conditions, including temperature (Hussein, 2008). The most suitable temperature for the rooting is 15-20°C for hardwood cuttings and approximately 25°C for softwood cuttings (Saranga and cameron 2007). The rooting rate of tetraploid *Robinia pseudoacacia* cuttings was high when the soil was heated, resulting in the production of adventitious roots both from cortical parenchyma cells at the junction of the pith rays and cortex and from callus tissue. However, when the soil was not heated, adventitious roots were produced from only calluses. In addition, adventitious roots appeared five days earlier in the heated treatment. Presumably, when there is an interaction between the applied auxin (IBA) and the ATPase located in the cell membranes, acidification of the cell wall environment occurs and some acid-unstable hydrogen bonds break down; subsequently, molecular cross links between the structural cell wall polysaccharides rupture and cell wall plasticity increases. This process occurs more readily when the growing medium is warm. When the temperature is raised, the walls of the living cells in the cambium, xylem, and phloem of cuttings tend to be flexible, allowing adventitious roots to break through the outer layer easily.

Conclusion

Heat treatment was proven to be an effective strategy for production of the tetraploid Robinia pseudoacacia by hardwood cuttings. There were no root primordia in the tissues of the cuttings before cutting. Adventitious roots of cuttings originated from callus tissue and the junction between the pith rays and cortical parenchyma cells with heat treatment but only from callus tissue without heat treatment. Additionally, the minimum effective accumulated temperatures for callus formation, adventitious root formation and root elongation of tetraploid Robinia pseudoacacia hardwood cuttings were 109.25°C, 211.68°C and 301.38°C, respectively.

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