

Root induction in radiata pine using *Agrobacterium rhizogenes*

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Financial support: Doctoral scholarship from the University of Canterbury to Mingshan Li.

Keywords: *Agrobacterium rhizogenes*, auxin, co-cultivation, *Pinus radiata*, rooting.

Root induction using *Agrobacterium rhizogenes* was conducted in hypocotyl explants, intact seedlings, de-rooted seedling cuttings and adventitious shoots of radiata pine (*Pinus radiata* D. Don). Use of two *A. rhizogenes* strains (A4T and LB9402), with or without application of IBA, can trigger root formation in different explants. Strain LBA9402 was more effective than A4T in increasing rooting percentage and root number. Addition of 4.4 μ MIBA to the medium further enhanced rooting from the cultured hypocotyl segments inoculated with the two *A. rhizogenes* strains. Strain LBA9402+IBA induced in about 75% of the cultured hypocotyl segments to form roots. In contrast the controls failed to initiate roots on intact seedlings or cultured segments in presence or absence of IBA. Rooting of adventitious shoots from 3 year-old radiata pine was improved following inoculation with LBA9402, suggesting that this rooting treatment has potential to aid clonal propagation of radiata pine.

Vegetative propagation of radiata pine (*Pinus radiata* D. Don), a species of considerably economic importance in several southern-hemisphere countries (Walter et al. 1994), plays an important part in commercial forestry in New Zealand. Unfortunately, like most conifers, some radiata pine explants and elite germplasms are particularly difficult to root, even with the application of auxins (De Klerk, 2002). With this problem in mind, recent investigations on rooting radiata pine have been carried out with the aim of uncovering useful biochemical markers associated with the process (Li and Leung, 2000; Li and Leung, 2001).

Before the late 1980s, it had been considered that only dicotyledonous plants could be infected by *Agrobacterium*

rhizogenes but not mono- or polycotyledonous plants (De Cleene and De Ley, 1981; Strobel and Nachmias, 1988). However, cross-inoculation tests have since shown that the possible host-range is much wider. Since the early 1990s reports have appeared on root induction by *A. rhizogenes* in monocotyledonous (Porter, 1991) and polycotyledonous plants (McAfee et al. 1993). *A. rhizogenes* has been used to enhance adventitious root formation in almond (Bassil et al. 1991; McAfee et al. 1993; Hatta et al. 1996), hazelnut (Bassil et al. 1991), pine and larch (McAfee et al. 1993), and jujube (Hatta et al. 1996). This bacterium transfers its transfer-DNA (T-DNA) which is a portion of the large plasmid called the root-inducing plasmid (pRi) to susceptible plant cells where the T-DNA, if integrated into the nuclear genome of the plant cell, will encode genes that direct the synthesis of auxin (indole-3-acetic acid) and / or increase the sensitivity of the transformed plant cells to auxin (McAfee et al. 1993; Hatta et al. 1996). The endogenous production of auxin and / or an increase in auxin sensitivity can lead to the formation of hairy roots at the site of infection (White et al. 1985).

This study reported the effects of two strains of *A. rhizogenes* on root formation in radiata pine. In particular, the potential for a good cultured hypocotyl system for investigations of adventitious root formation in pines has been demonstrated.

MATERIALS AND METHODS

Bacterial culture

Two strains of virulent *Agrobacterium rhizogenes*, A4T (Dommissie et al. 1990) and LBA9402, were used in this

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experiment. Strain A4T contains a root-inducing plasmid, pRiA4 in C58 chromosomal background. Strain LBA9402 is a rifampicin-resistant derivative of NCPPB1855 (Pomponi et al. 1983), which was kindly provided by Dr. D. Clapham (Swedish University of Agricultural Sciences, Uppsala). It harbors the root-inducing plasmid pRi1855 and the binary plasmid p35SGUSINT which carries genes coding for neomycin phosphotransferase II driven by the nopaline synthase promoter, and an intron-containing *uidA* gene coding for β -glucuronidase (GUS) driven by a cauliflower mosaic virus (CaMV) 35S promoter (Tzfira et al. 1996a; Tzfira et al. 1996b). The bacteria were cultured either on solid YM medium (Life Technologies, Gaithersburg, MD, USA) or in liquid YM medium at 26°C. When the bacterial suspension was used, its absorbance at 550 nm was adjusted to 0.4-0.5 (Tzfira et al. 1996a; Tzfira et al. 1996b).

Growth of seedlings

Seeds were collected in 1995 from a population of open-pollinated radiata pine grown in Canterbury, New Zealand. Seeds were surface-sterilized in 70% (v/v) ethanol for 30 s, rinsed briefly in sterile water; then soaked in 50% (v/v) of a commercial bleach (containing 31.5 g/liter active sodium hypochlorite) for 30 min before being rinsed thoroughly with sterile water. The sterilized seeds were sown in autoclaved vermiculite in tissue culture jars, and stratified in a cold room (4°C) for one week. The jars were then maintained in a warm dark room (26°C) till seedling emerged before transfer to a plant growth room at 22°C with continuous lighting at 80 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Uniform seedlings, which were about 1 mm in diameter and with 2.5-3 cm long hypocotyls, were selected for use as cuttings. All experiments here were on seedling materials grown aseptically unless indicated otherwise. In the appropriate experiment, non-aseptic seedlings were raised in the same way as described above, except that the seeds were not surface-sterilized, and sown directly in vermiculite in small containers (22 cm \times 15 cm \times 12 cm).

Inoculation of hypocotyl segments

Hypocotyl segments of about 1 cm long, two or three from each seedling, were randomly selected and inserted with the cut end proximal to the root into $\frac{1}{2}$ MS (Murashige and Skoog, 1962) medium (containing 20 gL^{-1} sucrose and 8 gL^{-1} agar) in Petri dishes. To the top cut-surface of the segments, a colony of the appropriate agrobacterial strain was inoculated with a loop, and then the petri dishes were placed in a plant growth room as described above. The segments without inoculation with the bacteria were designated as controls. The segments were either kept in the IBA (indole-3-butyric acid)-free medium or transferred to the above medium supplemented with 4.4 μM IBA (6-benzylaminopurine) at day 7 for 1 week before they were transferred back to the growth regulator-free medium. At various times during culture, the Petri dishes containing the

hypocotyls segments were placed under a stereomicroscope for observation.

Inoculation of de-rooted seedlings

The cut end of the de-rooted hypocotyls of axenically grown seedlings were either dipped in a bacterial colony grown on solid medium or co-cultivated with an appropriate agrobacterial suspension for 1 or 24 hrs, and then inserted upright with the cut end proximal to the root into autoclaved vermiculite saturated with $\frac{1}{2}$ GD (Gresshoff and Doy, 1972) nutrient solution (McAfee et al. 1993) in tissue culture jars. Subsequently, the jars were placed in a plant growth room as described above. Control cuttings were inserted in the vermiculite as described above either after the cuttings were made without dipping into a bacterial colony or after they were cultured in liquid YM medium for 1 or 24 hrs.

Inoculation of intact seedlings

Hypocotyls of non-aseptic intact seedlings were injected with approximately 10 ml of an appropriate agrobacterial suspension at the site of about 1.5-2.0 cm above the hypocotyl-root junction using a syringe fitted with a 24G needle. The needle was inserted into the hypocotyl and pushed through the centre. After this the containers were covered with a plastic film and placed in a plant growth room as described above. Control seedlings were treated in the same way except that the bacterial suspension was replaced with liquid YM medium.

Inoculation of adventitious shoots

Three year-old greenhouse-grown seedlings were pruned to remove the top 5-7 cm of growth and after 4 months the newly emerged adventitious shoots with w3 3-5 nodes were used for bacterial inoculation as follows. The adventitious shoots were injected *in vivo* with approximately 20 μl of the bacterial suspension using a syringe fitted with a 24G needle at the site of 10 - 15 cm from the tops of the adventitious shoots. One week later, the shoots were removed from the plants at about 0.5 cm below the injection site, and inserted upright in tap-water saturated vermiculite in small containers (22 cm \times 15 cm \times 12 cm) that were covered with a plastic film and then placed in a plant growth room as described above. Control shoots were treated in the same way except that the bacterial suspension was replaced with liquid YM medium. After 6 weeks following inoculation with LBA 9402, the newly formed roots were excised and assessed for GUS activity histochemically as described in Walter et al. 1994.

Statistical analyses

All experiments were repeated at least twice and similar trends of data were obtained. The number of hypocotyl explants or adventitious cuttings in each of the different treatments was 45. Data of percentages were either

converted by arcsin transformation prior to ANOVA (analysis of variance, $p < 0.05$) or evaluated for independence using Chi-square test. Multiple comparison test was performed using Duncan's multiple range test ($\alpha = 0.05$) following ANOVA. All statistical analyses were performed using the SPSS for Windows statistical software package (SPSS Inc., Version 8.0, 1998).

RESULTS

Rooting trials with hypocotyl segments

High survival rates were observed in this system and there was no significant difference among the treatments ([Table 1](#)). This could be due to the hypocotyl segments being cultured in tissue culture medium. The rooting percentages were significantly influenced by both bacterial strains and the supplement of IBA in the medium. Root numbers were also increased by addition of IBA, particularly in the treatment with strain A4T ([Table 1](#)). The hypocotyl segments co-cultivated with strain LBA9402 could produce a significantly greater rooting percentage and mean root number compared with those treated with A4T. Moreover, both the rooting percentage and mean root number in the treatments with the bacterium could be enhanced by the supplement with IBA in the medium ([Table 1](#)). However, no root formation resulted in cultured hypocotyl segments without inoculation with *A. rhizogenes*, regardless whether IBA was present or absent in the medium.

Under a stereomicroscope, morphological difference was not observed between the inoculation with A4T and LBA9402 10 days after inoculation. At this stage, a bacterial film appeared on the cut surface and the cells in the area close to differentiating resin ducts, where the root primordia were expected to be initiated according to our histological observations (Li and Leung, unpublished data). As early as day 14, primordia-like lumps could be observed in the treatment of LBA9402+IBA, but in the treatment of LBA9402 without IBA primordia were hardly seen at this stage. This suggests that addition of IBA to the medium seemed to enhance root formation earlier. The primordia in the treatment of LBA9402+IBA could be clearly observed at day 16. At the same time, only calli were observed in the controls without inoculation with the bacterium. One week later, the roots formed in the treatment of LBA9402+IBA were developing well. In contrast, only few primordia were observed in the treatment of LBA9402 without IBA. It seems that more calli were produced in controls at this stage regardless whether IBA was present or not in the medium. Surprisingly, the amount of calli formed did not appear to be very different between the two controls (with and without IBA in the absence of bacterial inoculation). At day 26, the newly formed roots developed further, and the longest root reached up to 2.3 cm.

Rooting trials with intact seedlings

After 6 weeks, the survival of seedlings co-cultivated with

strains A4T or LBA9402 and the control, was similarly high ([Table 2](#)). More adventitious roots were formed in response to inoculation with LBA9402 than A4T. Root formation was not observed in the control ([Table 2](#)). Adventitious roots frequently formed from the infection site ([Figure 1A](#) left seedling), but roots appearing from above and below the infection site were also observed in some inoculated seedlings ([Figure 1A](#) the second seedling).

Rooting trials with de-rooted seedlings and adventitious shoots

Survival of the de-rooted seedlings was very poor ([Table 3](#)). The few roots formed in the de-rooted seedlings were found at the very bottom of the cuttings ([Figure 1B](#)). Strain LBA9402 was the only strain used to induce roots in adventitious shoots because it seemed to be more effective than A4T in the preliminary root induction experiments. Eight weeks after inoculation, more adventitious shoots inoculated with LBA9402 rooted than control shoots did (1.6 ± 0.1 and 0.67 ± 0.33 , respectively). Rooting percentages between the two treatments were significantly different at $p < 0.01$ using Chi-square test of independence. Also, the adventitious shoots inoculated with LBA9402 ([Figure 1C](#)) produced a significantly greater mean number of roots compared to control (54.8 ± 9.7 and 8.0 ± 4.0 , respectively).

DISCUSSION AND CONCLUDING REMARKS

The results here show that different experimental protocols could significantly affect survival percentages and the subsequent rooting percentages and root number. For instance, the survival and rooting was poorest in the experiment with de-rooted seedling cuttings kept in moist autoclaved vermiculite. The low survival rates may be due to the de-rooted seedlings not being able to absorb water and nutrients effectively from the culture medium since large pieces of vermiculite formed large spaces and the cut surfaces sometimes failed to touch the vermiculite.

Interestingly, when hypocotyl segments inoculated with either A4T or LBA9402 were cultured in medium supplemented with IBA, significantly higher rooting percentage and root number were observed than when they were cultured in the medium without IBA ([Table 1](#)). However, exogenous auxin was not required for the *A. rhizogenes* strains to induce higher rooting percentages and root number in the hypocotyls of intact seedlings or adventitious shoots, compared with the hypocotyl segments inoculated with these strains alone and cultured in the medium without IBA ([Table 1](#) and [Table 2](#)). This suggests that in this system the endogenous auxin level might not be high enough for root formation due to the hypocotyl segments devoid of shoot apex, therefore, the exogenous supply of IBA might augment the auxin produced as a result of the action of the auxin biosynthesis genes of *A. rhizogenes* (Liu et al. 2002). It is possible that the hypocotyl segments inoculated with the bacterium were

more sensitive to increased auxin supply since *A. rhizogenes* is known to encode genes that increase auxin sensitivity to plant tissue (McAfee et al. 1993; Hatta et al. 1996). While the reason for the synergistic action of IBA and *A. rhizogenes* is not clear, the best rooting response (approximately 75%) in this study was obtained when the hypocotyl segments were inoculated with LBA9402 before they were cultured in semi-solid ½ MS medium supplemented with 20 g/l sucrose and 9 mg/l IBA. McAfee et al. 1993 and Hatta et al. 1996 also found that different strains of *A. rhizogenes* brought about different rooting responses in pine (*Pinus*) and larch (*Larix*) spp., and in jujube (*Ziziphus jujuba*), respectively.

In this study, the adventitious roots induced in various *A. rhizogenes* treatments could not survive on hormone-free medium in several attempts. β-Glucuronidase (GUS) activity was also not detected in the roots formed in the treatment with LBA9402 which carries this reporter gene. McAfee et al. 1993 did not detect T-DNA in the induced roots of *Pinus monticola*, *P. banksiana* and *Larix laricina* using the left T-DNA region of pRiA4b as DNA probe. The authors suggested that no T-DNA transfer had occurred or that it was present at a level lower than that detectable by the Southern blot procedure. Bassil et al. 1991 were unable to determine if the roots induced on hazelnut cuttings contained transformed tissue after opine and molecular analysis for TR- and TL-DNA. In these cases, root induction was probably due to an improvement of rooting environment by the bacterium (McAfee et al. 1993; Simpson et al. 1986). Recently, similar results were obtained by Falasca et al. 2000 who conducted an experiment to investigate how *A. rhizogenes* triggers *de novo* root formation in a recalcitrant walnut plant. They found that rooting on the infected cuttings was enhanced by IBA, which accelerated and increased root meristemoid formation, in comparison with the treatment without hormone. PCR and Southern blotting analyses showed that root meristemoid formation was not accompanied by genetic transformation.

The cultured hypocotyl segments described here is a good system for the study of adventitious root induction in pines by *A. rhizogenes*. Since the process of root formation can be easily manipulated under highly defined experimental conditions, and the rooting response could be relatively high, this makes this system attractive for further investigations into the mechanism of root induction by *A. rhizogenes* in pines. Strain LBA9402 significantly increased rooting percentage and root number in adventitious shoots. It seems worthwhile to screen additional *A. rhizogenes* strains for efficient root induction in pines. This application, possibly combined with other treatments, appears to be useful for vegetative propagation of radiata pine clones that are difficult to root.

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APPENDIX

Tables

Table 1. The effects of bacterial strains and IBA on root formation of *P. radiata* hypocotyl segments upon culture for 6 weeks. Mean values \pm SE from all six treatments of each of the three parameters (% of survival, % of rooting and No. of roots/cutting) were compared using Duncan's multiple range test ($\alpha=0.05$) following ANOVA at $P<0.05$ (p-values were 0.222, 0.016 and 0.003, respectively). Those means that were different among the different treatments of a parameter are denoted with a different letter. Data of percentages were converted by arcsin transformation prior to ANOVA. Control = hypocotyl segments not inoculated.

Strains	IBA	No. of cuttings	Survival (%)	Rooting* (%)	No. of roots/cutting**
A4T	+	45	91.1a	33.8b	1.81a
	-	45	88.9a	4.9d	0.67b
LBA9402	+	45	93.3a	76.5a	2.40a
	-	45	84.4a	18.6c	1.67a
Control	+	45	97.8a	0.0d	-
	-	45	95.6a	0.0d	-

* = average percentage.

** = average number of roots formed per rooted cutting.

Table 2 Root formation of intact *Pinus radiata* seedlings after 6 weeks from injection with *A. rhizogenes* strains. Mean values \pm SE from all treatments of a parameter were compared and those means denoted by the different letter are significantly different ($P<0.05$) using Duncan's multiple range test following ANOVA (p-values for % survival, % rooting and No. of rooting/cutting were 0.531, 0.01 and 0.006, respectively). Data of percentages were converted by arcsin transformation prior to ANOVA. Control = intact seedlings injected with YM medium only.

Strains	No. of seedlings	Survival (%)	Rooting* (%)	No. of roots/seedling**
A4T	45	84.4a	39.5b	1.67b
LBA9402	45	75.6a	59.6a	2.47a
Control	45	77.8a	0.0c	-

* = average percentage of cuttings that survived and formed roots at the end of the experiment.

** = average number of adventitious roots formed per seedling.

Table 3 Effect of two *A. rhizogenes* strains on survival and root formation of de-rooted seedlings of *Pinus radiata*. Mean values \pm SE from all treatments of a parameter were compared and those means denoted by the different letter are significantly different ($P < 0.05$) using Duncan's multiple range test following ANOVA (p-values for % survival, % rooting and No. of roots/cutting were 0.676, 0.014 and 0.230, respectively). Data of percentages were converted by arcsin transformation prior to ANOVA. Control = de-rooted seedlings without inoculation with the agrobacterium.

Inoculation	Strains	No. of cuttings	Survival (%)	Rooting* (%)	No. of roots/cutting**
Colony	A4T	45	24.4a	0.0b	-
	LBA9402	45	22.2a	0.0b	-
	Control	45	28.9a	0.0b	-
Suspension 1 hr	A4T	45	26.7a	17.8a	1.5
	LBA9402	45	20.0a	0.0b	-
	Control	45	22.2a	0.0b	-
Suspension 24 hrs	A4T	45	28.9a	0.0b	-
	LBA9402	45	33.3a	11.1ab	1.5
	Control	45	26.7a	0.0b	-

* = average percentage of cuttings that survived and formed roots at the end of the experiment (after six weeks from bacterial inoculation).

** = average number of roots formed per rooted cutting.

Figures

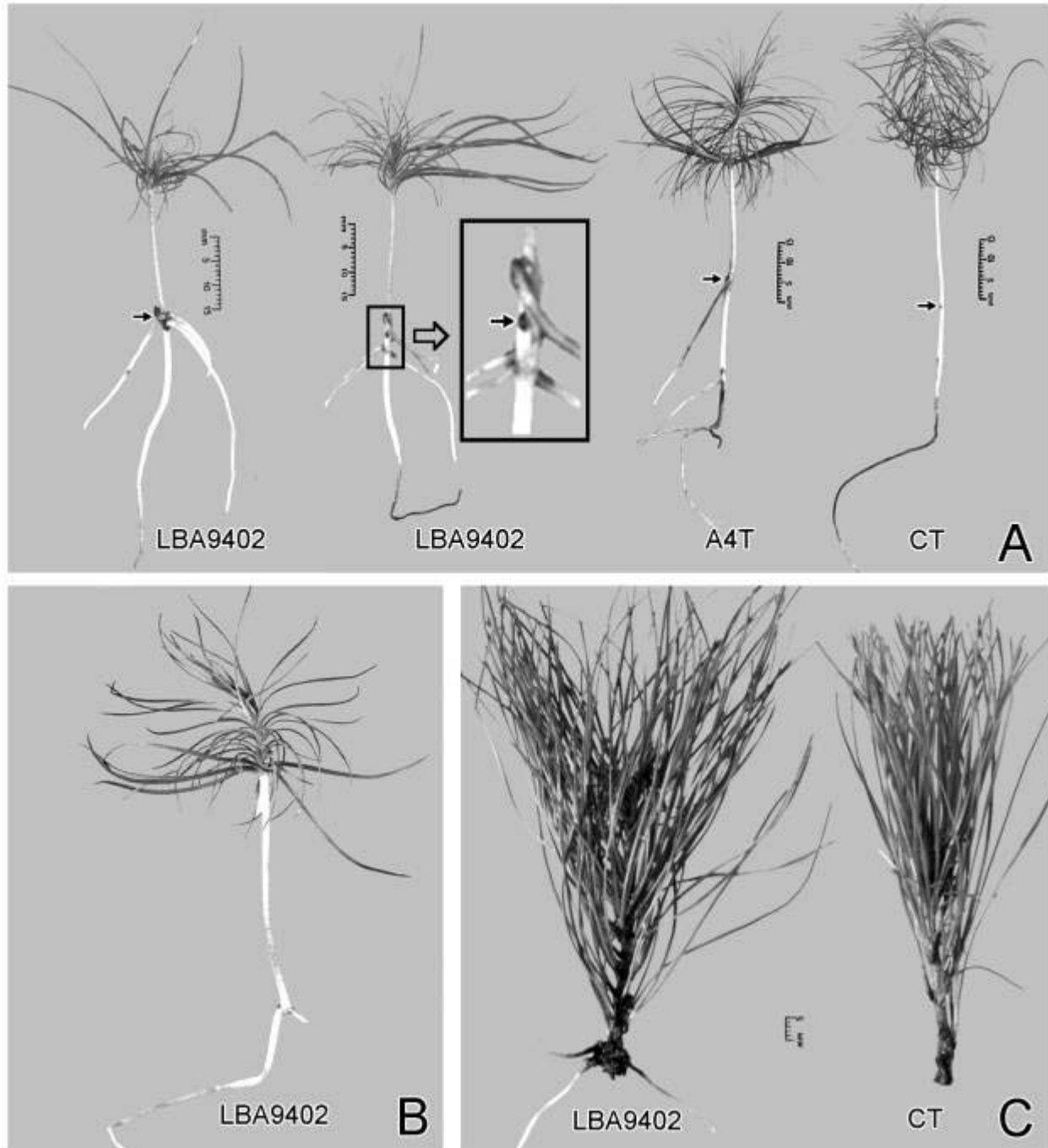


Figure 1. Root formation induced by *A. rhizogenes* in intact seedling hypocotyls, de-rooted seedling cuttings, and adventitious shoots. (A) In intact seedlings, roots induced by strain LBA9402 and A4T. Arrows indicate injection sites. CT = control seedling. (B) Roots induced by strain LBA9402 in a de-rooted seedling cutting. (C) Roots induced by strain LBA9402 in an adventitious shoot and a control shoot (CT).