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# Effect of Seasons, Gender and *Agrobacterium rhizogenes* Strains on Adventitious Root Induction of Male and Female *Juniperus communis* L.

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Abstract

Scrutinizing of different aspects in vegetative propagation of Iranian *J. communis* L. is of prime importance in order to prevent extinction of such valuable conifer. In this research, the effects of different seasons, gender, different chemical compounds (IBA, PBZ, putrescine and sodium nitroprusside) and three strains of *Agrobacterium rhizogenes* on *ex-vitro* rooting capacity of male and female *J. communis* L. were evaluated. Rooting of semi-hard wood *J. communis* L. cuttings in autumn were notably successful either in female or male plants. Research results indicated that cutting gained in September provides the highest rooting percentage in female plants while no significant differences in rooting ability were found in male plants which were collected in December and September. There were a female-biased root induction trends between genders. Treatment of female cuttings with IBA declined rooting to the half, even in male cuttings it completely suppress root induction ability compared to control plants. Our empirical evidence on presence of genetic difference in random amplified loci of male and female plants to some extent can explain the difference in rooting competency which can be influenced by difference in genetic make up. A4 strain of *A. rhizogenes* managed to yield 53.5% rooting compared to the other strains in male plants. The gender-mediated adventitious root formation of *J. communis* L. which has supported with some genetic evidence will pave the way for further fundamental studies to delineate the missing molecular key of rooting mechanisms.

**Keywords:** Adventitious rooting, *Agrobacterium rhizogenes*, Common juniper, Dioecy, Season.

Abbreviations: IBA: Indole-3-butyric acid, ISSR: Inter simple sequence repeat, MNR: Mean number of roots, MRL: Mean root length, PBZ: Paclobutrazol, PGR: Plant growth regulators, SBP: Shoot burn percentage.

## INTRODUCTION

Conifers provide great part of world's timber and oxygen. Their evergreen feature made them even more valuable than other woody plant species. They are common ornamentals in public and private gardens. Apart from their ecological and aesthetic values, they have been using for production of different natural products suitable for different industry (Sarmast, 2016). Juniper is one of the most scattered species among conifers with extreme flexibility to harsh environmental condition. *Juniperus communis* L. grows in Alborz mountain chains. Their ground cover habit can protect topsoil from erosion and drought. Unfortunately, in recent years – due to the anthropogenic disturbances, low natural propagation and exceeded drought condition – population of this valuable endangered species declined steeply. Therefore, it is necessary to consider variable approach for improving clonal propagation of this species. There are an increasing number of academic literatures addressing the anticancer and anti-microbial activities in extract obtained by different aerial parts of *J. communis* L. Carpenter *et al*. (2012) validated the presence of anti-mycobacterial activity in aerial part extract of *J. communis* L. Moreover, the anti-cancer natural products produced by endophytic fungus of *J. communis* L. are also a great area of study (Kusari *et al*., 2009). Repelled compound against ticks and mosquitoes were also reported by Carroll e*t al*. (2011).

According to the above statements, propagation of this species is of prime importance in order to impede extinction of this endangered species.

The big dilemma facing commercial production of most conifer species is ineffectiveness of adventitious root induction ability of these species (Sarmast, 2018). There are only very few reports on vegetative propagation of *J. communs* L. if any. In one attempt on vegetative propagation of *J. excelsa* Bieb., it has been shown that IBA and harvested cuttings in winter and fall had influential effects on rotting percentage (Rifaki *et al*., 2002). In *J. virginiana*, rooting has improved when winter collected stem cuttings exposed to 5000 mg $L^{-1}$ IBA.

The potent effect of *Agrobacterium rhizogenes* on the root induction of most coniferous trees has been reported in earlier researches. In one report, *A. rhizogenes* strain A4, has increased rooting percentage of *Pinus maximartinezii* Rzedowsky and *Pinus pinceana* Gordon up to 65% and 67%, respectively (Villalobos-Amador *et al*., 2002). Combination of IBA and *A. rhizogenes* managed to improve rooting efficiency of *Araucaria excels* (Sarmast *et al*., 2012) in MS medium. Root induction of *Pinus monticola* Dougl. was also achieved through co-cultivation with A4 strain of *A. rhizogenes* (McAfee *et al*., 1993). To the best of our knowledge the research on vegetative propagation of *J. communis* L. is seldom. In addition, *A. rhizogenes*-dependent adventitious root formation of *J. communis* L. has never previously been reported. Most recent works on aforementioned species dealt with the subject of medicinal activity of its natural products. However, this area of research without having enough knowledge on how to propagate this plant species is purposeless. The objective of the present work was to evaluate the seasons, IBA, PBZ, sodium nitroprusside, putrescene, gender and different *A. rhizogenes* strains effects on adventitious root induction ability of *J. communis* L.

## MATERIALS AND METHODS

### Plant materials

All the stem cuttings of *Juniperus communis* L. which were used in this research, directly collected from Chahar Bagh in the Central District of Gorgan County, Golestan Province, Iran (located at 36° 35′ 17" N, 54° 27′ 29" E, and 2230 m above sea level). Due to the great genetic diversity among *J. communis* L. populations, we have tried to select one male and female elite tree for adventitious root induction experiment. Female plants were detectable through small green and purple cones. These freshly collected samples transferred to the greenhouse for performing different experiments.

**Effects of seasons and chemical compounds**

Proximal end-wounded *J. communis* L. cuttings with the length of 15 cm were immersed for 10 min in 5000 mg $l^{-1}$ iprodione + carbendazim (50%) to control fungal contamination. All the leaves and lateral branches at the proximal-end of stems were removed and wounded longitudinally.

In one experiment, cuttings were separately collected in March, May, September and December from male and female plants so as to shed light on seasonal effects on root induction ability.

IBA at concentrations of 2500 and 7000 mg $L^{-1}$; PBZ at concentration of 4 mM; putrescine at concentration of 2000 mg $L^{-1}$ and sodium nitroprusside a donor of nitric oxide at concentration of 0.3 mM exposed to the proximal end of cuttings. In another experiment, the combination effects of IBA and PBZ and also combination of IBA and putrescine with aforementioned concentrations were applied to the stem cuttings. Apart from IBA which applied as a quick deep, stem cuttings were exposed to other chemicals for 10 min. Control stem cuttings kept in pure deionized water for 10 min. Soon after applying different treatments, stem cuttings were cultured in pre-sterilized peat and perlite (1:1 in ratio) soil mixture under a mist system that operated 3 times in every 1 h in summer and one time in every 1 h in winter. Misting lasted 30″ each time. Bottom heat also ran at a temperature of 27 ºC.

***A. rhizogenes* treatments**

Three wild strains of *A. rhizogenes* were chosen to examine their ability for induction of adventitious roots on *J. communis* L. stem cuttings under greenhouse condition. Grown bacteria in liquid LB media (tryptone 10 g $L^{-1}$ NaCl 5 g $L^{-1}$, yeast extract 5 g $L^{-1}$) centrifuged in 3000 rpm for 10 min to harvest cells. Optical density of bacterial culture was measured at 600 nm ($OD_{600}$) and then adjusted to 0.5 with sugar-free MS medium. The base of wounded stem cuttings was immersed in this medium overnight (for 10 h). The inoculation medium supplemented with 100 µM of acetosyringone. Exposed cuttings to *A. rhizogenes* were sown into the soil mixture in a greenhouse. In addition to male and female *J. communis* L., *J. sabina* was also used in separate experiment in order to examine the effects of A4 *A. rhizogenes* on aforementioned three samples.

**Genetic analysis**

To examine the genetic background differences between male and female *J. communis* L., we take advantage of ISSR markers. Four ISSR markers ((GA)8YC-2, (GT)8YC-6, (GA)8C-16 and (GA)9A-20) were selected to replicate *J. communis* L., DNA in this experiment. Leaf samples for DNA extraction were chosen from four separated male and female native *J. communis* L. of the region. We have marked the male and female *J. communis* L. that we have used for rooting experiment. The experiments were carried out according to the protocol described previously (Sarmast *et al*., 2015). We amplified 4 male and female *J. communis* L. DNA samples with four ISSR markers via thermocycler.

**Experimental design**

In case of adventitious root formation, each treatment had three replications. The number of stem cuttings in each replication was 18. Collection of data was ensued 60 days after planting the stem cuttings in soil mixture. Rooting was evaluated based on mean number of shoots (MNS), mean root length (MRL), and shoot necrosis percentage (SNP) along with rooting percentage. Regarding to the genetic analysis, reproducible PCR-amplified fragments on the gels were used to show differences between male and female plants. PCR-amplified fragments on the gels were scored as 1 or 0 for the presence or absence of DNA fragment. This experiment was carried out as a completely randomized design. Data were analyzed using RSudio (R version 3.4.2) statistical software, and means were compared with LSD and T-Test (P < 0.05).

## RESULTS

### Role of seasons

To examine the relationship between different seasons and root induction ability of female *J. communis* L. plants, semi-hard wood stem cuttings of *J. communis* L. were cultured in soil mixture inside of greenhouse in different seasons. Wounded cuttings were taken in September managed to yield the highest rooting percentage which was not significantly different from the samples collected in December (Fig. 1). All the collected stem cuttings in May were unable to produce roots. Stem cuttings which were collected in September maintains their green leaves and had a high quality compared to the other seasons. Cuttings collected in September had the highest mean root length (Table 1).

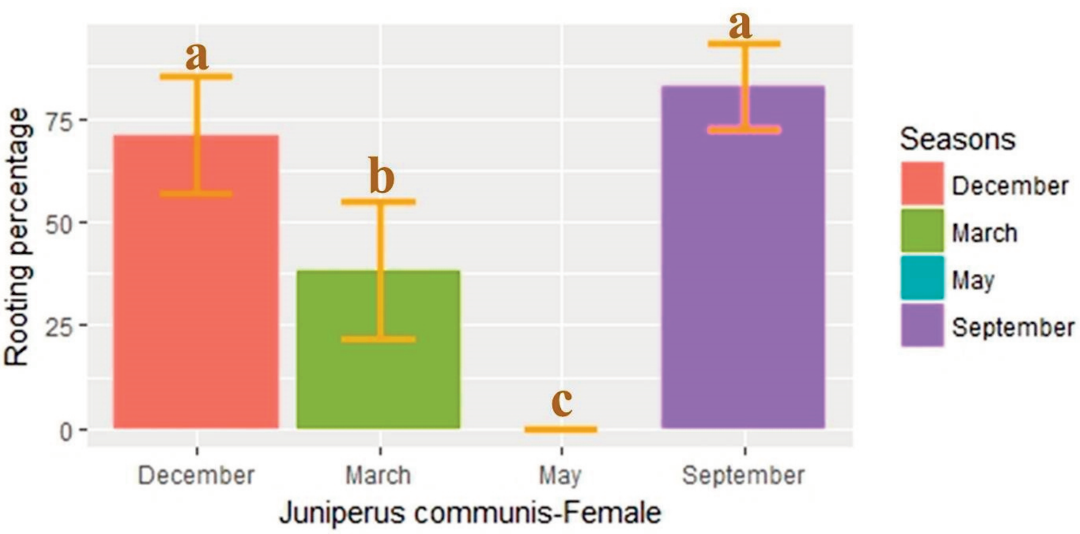


Fig.1. Variation in root induction ability of female *J. communis* L. throughout the year.

Table 1. Role of season on root induction ability of female *J. communis* L.

| Seasons | MNR | MRL (mm) | SNP† |
|---|---|---|---|
| March | 0.47b* | 3.10b | 0.70ab |
| May | 0.00b | 0.00b | 1.40a |
| September | 2.63ab | 68.63a | 0.00b |
| December | 5.16a | 4.43b | 1.80a |

* In each column means with the same letters are not significantly different according to the LSD test at P< 0.05 in R program.
† Shoots necrosis: 3 is the highest necrosis rate of shoot leaves and zero represents no burning.
Mean number of roots (MNR), Mean root length (MRL), Shoot necrosis percentage (SNP).

Regarding to the male *J. communis*, no significant difference was found in rooting percentage in September and December. Generally, male cutting collected in May were not able to produce root during the course of experiment (Table 2).

Mean root length of both male and female *J. communis* progressively increased in September collected samples.

Table 2. Role of season on root induction ability of male *J. communis* L.

| Seasons | Rooting (%) | MNR | MRL (mm) | SNP† |
|---|---|---|---|---|
| March | - | - | - | - |
| May | 0.00b* | 0.00b | 0.00b | 0.99ab |
| September | 23.35a | 0.37b | 13.34a | 0b |
| December | 31.56a | 1.45a | 1.91b | 1.28a |

* In each column means with the same letters are not significantly different according to the LSD test at $P < 0.05$ in R program.
† Shoots necrosis: 3 is the highest necrosis rate of shoot leaves and zero represents no burning.
Mean number of roots (MNR), Mean root length (MRL), Shoot necrosis percentage (SNP).

## Comparing male and female adventitious root induction ability

The results provided by T-test in R program showed that the rooting ability of female *J. communis,* to great extent is higher than male plants. Rooting percentage in September and December in female *J. communis* is three to two times of male plants, respectively. Amplification of male and female Juniper DNA by four ISSR markers also showed genetic difference in amplified loci (Table 3; Fig. 2).

Table 3. T-test experiment between male and female *J. communis* L.

| Season | Female | Male | *P-value* |
|---|---|---|---|
| May | 0.00* | 0.00 | 0.00 |
| September | 82.60 | 23.35 | 0.0024 |
| December | 71 | 31.56 | 0.0034 |

T.test was carried out with R software program. * Stem cuttings were unable to produce roots.

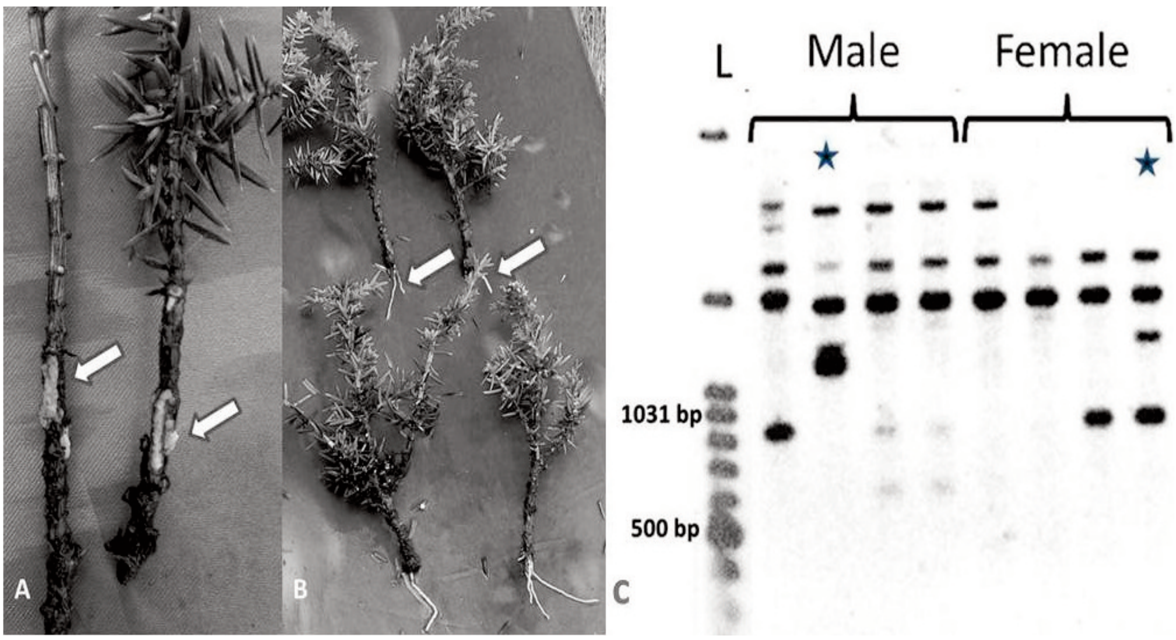


Fig. 2. A. Wound-induced callus of *J. communis* L. B. Root production of Male *J. communis* L. stem cuttings which were collected in September. C. DNA fingerprinting of male and female *J. communis* L. by ISSR marker. Marked line with star represent plants which were used in rooting experiment.

### Effects of IBA, Sodium nitroprusside, PBZ and putrescine

Sodium nitroprusside (nitric oxide donor), PBZ and putrescine were not able to improve rooting ability of *J. communis* L. compared to control plants (data not shown). Interestingly, male *J. communis* L. rooting totally suppressed when exposed to 2500 mg $l^{-1}$ IBA. In female *J. communis* L., rooting ability diminished to half as compared to untreated control (Table 4). Regardless of gender, the toxicity effects of IBA on *J. communist* L. were clear.

Table 4. Effects of IBA on root induction ability of male and female *J. communis* L.

| **Treatments** | **Rooting (%)** | **MNR** | **MRL (mm)** | **SNP†** |
|---|---|---|---|---|
| Male-control | 23.35c* | 0.37c | 13.3bc | 0.00b |
| Male + 2500 mg $l^{-1}$ IBA | 0.00d | 0.00c | 0.00c | 1.3a |
| Female-control | 68.6a | 2.6a | 68.6a | 0.00b |
| Female + 2500 mg $l^{-1}$ IBA | 30b | 1.5b | 30b | 0.24b |

* In each column means with the same letters are not significantly different according to the LSD test at P<0.05 in R program.

† Shoots necrosis: 3 is the highest necrosis rate of shoot leaves and zero represent no burning.

Mean number of roots (MNR), Mean root length (MRL), Shoot necrosis percentage (SNP).

### Role of *Agrobacterium rhizoenes* strains on juniper rooting

In this experiment, we exposed male *J. communis* L. semi-hard wood cuttings to three different strains of A4, 15834 and 01344 *A. rhizogenes* in order to improve its rooting efficiency and find the most usefulness strains. No statistically differences in mean number of roots were found in three different strains of *A. rhizogenes*–exposed cuttings. Nonetheless, A4 yields to a 53% rooting, a 20% more rooting compared to the untreated control (Table 5). A4-treated stem cuttings also showed the highest MNR and MRL, but this increase was not significantly different. The leaves of A4-treated plants were fairly healthy. In the follow up study we have decided to expose stem cuttings of male and female *J. communis* L. and *J. sabina* to A4 strain. Both gender of *J. communis* L. had the same ability for adventitious root formation, whereas A4-mediated root induction in *J. sabina* was nearly one-third of *J. communis* L. (Table 6).

Table 5. Effects of different strains of *Agrobacterium rhizogenes* to improve rooting capacity of male *J. communis* L.

| ***A. rhizogenes* strains** | **Rooting (%)** | **MNR** | **MRL (mm)** | **SNP†** |
|---|---|---|---|---|
| control | 31.56c | 1.45a | 1.46bc | 1.28a |
| A4 | 53.48a | 2.57a | 2.57a | 0.66a |
| 15834 | 36.56c | 1.33a | 1.33c | 0.63a |
| 01344 | 46.43b | 2.33a | 2.33ab | 0.67a |

* In each column means with the same letters are not significantly different according to the LSD test at P <0.05 in R program.

† Shoots necrosis: 3 is the highest necrosis rate of shoot leaves and zero represents no burning.

Mean number of roots (MNR), Mean root length (MRL), Shoot necrosis percentage (SNP).

Table 6. Exposure of A4 to two species of *J. communis* L. *J. Sabina*.

| Species | Rooting (%) | MNR | MRL (mm) | SNP[†] |
|---|---|---|---|---|
| Male *J. communis* L. | 53a* | 2.3a | 1.83a | 0.66b |
| Female *J. communis* L. | 45.8a | 3.37a | 2.87a | 1.79a |
| *J. sabina* | 16.7b | 0.83a | 1.00a | 1.07b |

* In each column means with the same letters are not significantly different according to the LSD test at P <0.05 in R program.

[†] Shoots necrosis: 3 is the highest necrosis rate of shoot leaves and zero represents no burning.

Mean number of roots (MNR), Mean root length (MRL), Shoot necrosis percentage (SNP).

## DISCUSSION

Propagation by stem cutting is a key tool for vegetative propagation which basically needs adventitious roots production to further growth as a compulsory need for successful cutting propagation. Improving the rooting success, to great extent contributes to diminish an excessive labor cost of propagation (Hartmann *et al.*, 2011). Apart from molecular key of rooting, other aspects of rooting are well addressed by different authors (Ragonezi *et al*., 2010; Hartmann *et al*., 2011; Wiesman *et al*., 1995; Davis and Haissig, 1990). Effects of different chemical agents on root induction were previously reported on conifers (Ragonezi *et al*., 2010). IBA as a PGR is a universal root inducing agent in *in vitro* and *ex-vitro* conditions (Davis and Haissing, 1990). Polyamines such as putrescine were found to be involved in mitotic cell cycles process required for rooting (Ragonezi *et al*., 2010).

As opposed to putrecine or spermidine which increases rooting frequency of *Pinus virginiana*, a 6.7% decline in rooting has reported by application of 0.001 mM spermine (Tang and Newton, 2005). The concomitant use of PBZ – a gibberellin synthesis inhibitor – and IBA, to great extent improve *Pinus caribaea* var. hondurensis rooting ability (Henrique *et al*., 2006).

Nitric oxide–mediated auxin response during adventitious root formation also reported by different researchers (Pagnussat *et al*., 2003; Gergoff Grozeff *et al*., 2018). However application of putrescine, PBZ and SNP did not have any significant effects on *J. communis* L. rooting percentage when compared to untreated control plants. Some wounded cuttings that were able to produce callus throughout the experiment were unable to produce roots which validate this idea that the callus production on cutting is not necessarily a prerequisite for root induction (Hartmann *et al*., 2011). Unexpectedly, we have seen negative effects on adventitious root formation when cuttings exposed to 2500 mg $l^{-1}$ IBA by quick dip. Rooting was completely ceased in male *J. communis* L. however, female plant rooting ability declined to the half amount of control plants. This likely due to the fact that even 2500 mg $l^{-1}$ is beyond its limits of tolerance. The negative effect of high concentration of IBA on root formation of *J. communis* var. depressa was previously reported (Houle and Babeux, 1994).

Due to the fact that female plants to some extent are easily to root compared to the male plants, IBA could not completely suppressed its root formation. These suppression effects of IBA on rooting in male and female *J. communis* L. likely depends to the endogenous concentration of the PGRs. Looking into the difference between male and female *J. communis* L. root induction ability, revealed a 3 and 2 times more root induction ability in female plant compared to male in September and December, respectively. This difference in adventitious root formation most likely is due to their genetic background difference which may result in production of different components in male and female plants. To test whether this manifest difference in rooting percentage associate with genetic differences between male and female *J. communis* L., we exploited a molecular marker based study to address this possibility. Amplification of ISSR markers in DNA extracted from leaves of male and female *J. communis* L. gave empirical evidence on presence of genetic difference in amplified loci.

However, these results could not rule out the possibility of direct connection between amplified loci and rooting ability, but the reported adventitious root induction ability to some extent can be explained by general genetic differences. The female-biased root induction in dioecious conifers like *J. communis* var. depressa was previously reported (Houle and Babeux, 1994). Among different seasons, September gave the better results for adventitious root formation. Our results are affirmative of the likewise study on the *ex-vitro* rooting of *J. excelsa* by Rifaki *et al.* (2002), who obtained higher adventitious root induction in autumn and winter.

In addition, the effects of different *A.rhizogenes* strains on male and female *J. communis* L. were also evaluated. Among those, A4 strain augmented the rooting percentage of male junipers up to 53%, a 22% significant increase compared to the untreated control. This is while the female plants could yield an average number of 43% in rooting. However, no significant differences in mean number of roots and mean root lengths were found among *A. rhizogenes* strains. It is noteworthy that the regenerated roots from male *J. communis* which were exposed to A4, appeared to be healthy and robust, with negligible signs of necrosis if any.

It is evident that the prolong exposure of cuttings to A4 strain of *A. rhizogenes* can substantially promote adventitious root induction in male *J. communis* L. This is consistent with the study by Villalobos-Amador *et al*. (2002) who has reported that micro-shoots of *Pinus maximartinezii* Rzedowsky and *Pinus pinceana* Gordon were able to increase rooting percentage up to 65% and 67%, respectively when exposed to A4 strain of *A. rhizogenes in vitro*. *A. rhizogenes*–mediated rooting reported in *Araucaria excelsa* R. BR. (Sarmast *et al*., 2012) and also in *Pinus monticola* Dougl. (McAfee *et al*., 1993).

Assessment of adventitious root formation through *A. rhizogenes* in *J. communis* L. has never previously addressed. The empirical results gained by this experiment accentuated the role of gender-mediated rooting in *J. communis* L. and shows there is a fundamental molecular mechanism behind this behavior which has yet to be found. In addition to that, the ineffectiveness of different chemical compounds and PGRs so as to triggering root formation will open a path for further research. Moreover, the role of A4 strain of *A. rhizogenes* among other wild strains needs additional attention for clonal propagators.

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