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In vitro shoot proliferation and enhancement of rooting in *Melocanna bambusoides* Trin. from nodal explants

W. Sayanika Devi and G. J. Sharma*
Department of Life Sciences, Manipur University, Imphal-795003, India

Abstract: An efficient protocol for *in vitro* propagation of *Melocanna bambusoides* Trin. is reported. Single nodal segments obtained from mature field grown culm collected during monsoon were tested for bud-break and shoot growth on Murashige and Skoog (MS) medium supplemented with different concentrations of 6- benzylaminopurine (BAP). The early bud-break was obtained on 2-5 mg l^{-1} BAP with 1-3 shoots per explants. Axillary shoots produced were multiplied on MS medium supplemented with 3-5 mg l^{-1} BAP in liquid medium. Combination of 2 mg l^{-1} kinetin (Kn) with 2 mg l^{-1} BAP enhanced the multiplication rate. *In vitro* rooting was carried out in half-strength MS medium. Addition of optimum concentration of 10 mg l^{-1}coumarin to 3 mg l^{-1} 3- indoleacetic acid (IAA) and 3 mg l^{-1} 3- indolebutyric acid (IBA) enhanced the rooting percentage. More than 80 per cent of the shoots could be rooted in half-strength MS medium containing 3 mg l^{-1} IBA, 10 mg l^{-1} coumarin and 0.05 mg l^{-1} BAP before transferring them to a semi-solid rooting medium. Synergistic interaction between an optimum concentration of 10 mg l^{-1} coumarin with auxins stimulates root growth and development. After a period of *in vitro* hardening, the propagules were acclimatized and then transferred to the soil with more than 80 per cent success.

Keywords: Axillary shoot multiplication, *Melocanna bambusoides*, micropropagation, *in vitro* bud-break, root induction.

INTRODUCTION

The Indo-Burmese region, which includes Manipur in the north-eastern India, being located at the confluence of the Burmese and the Indian tectonic plates, has been the 'Vavilovian centre of origin' for many cultivable crops. Of the total geographical area of Manipur, only 3.2 per cent represents dense forests. Bamboo is one of the eco-friendly crops, which is extensively found in these natural forests.

Melocanna bambusoides Trin. (locally Mulli/Moubiwa) is an important bamboo species. This bamboo has established well in the south-western region especially along the Jiri-Barak drainage because of its remarkable capacity to spread over the jhum land. It is a major source of raw material for paper industry because of high

* To whom correspondence should be addressed; E mail: gjs1951@ rediffmail.com, gjs1951@yahoo.co.in

content of alpha cellulose. It is also highly utilized by local people especially in the handicraft industry and house constructions. Besides, the young tender shoots are edible and possess high nutritional values. Bamboo shoots are important sources of phytosterols (Srivastava, 1990). The seeds of *M. bambusoides* are very large, fleshy and eaten by the tribal people during famine. However, this valuable resource is dwindling due to tremendous anthropogenic pressures. Incidence of gregarious flowering *of M. bambusoides* leads to death of entire culms after shedding seeds. Therefore, it is necessary to conserve this rich resource for its sustainable development. Bamboo is propagated traditionally by using seeds, offsets or rhizomes and also by culm cuttings and layering (Banik, 1994). Propagation by seeds is non-reliable due to long and unpredictable flowering cycles with some species flowering gregariously at species-specific intervals (Jansen, 1976). Moreover, the vegetative propagation requires considerable time and labour for large-scale plantations. Tissue culture techniques offer the most suitable alternative method for rapid propagation of this potentially promising species. Few reports are available on clonal propagation of bamboo culms such as *D. strictus* (Chaturvedi *et al.*, 1993), *D. giganteus* (Ramanayake and Yakandawala, 1997), *B. vulgaris* (Hirimburegama and Gamage, 1995; Ramanayake *et al.*, 2006) and *B. bambos* (Arya and Sharma, 1998). However, there are no reports on the development of protocols on the successful tissue culture techniques for *M. bambusoides*. This paper describes a suitable protocol for successful initiation of cultures, rapid proliferation of axillary shoots and high rooting percentage by employing nodal explants of field-grown culms of known age.

MATERIALS AND METHODS

Culture initiation

Single nodal segments with unsprouted buds (2.5-3.0 cm) collected during the onset of monsoon (June-August), from young primary and lateral branches of 1-year-old field grown clump of *M. bambusoides* were used to initiate cultures. The nodal segments were first cleaned with cotton swab dipped in dehydrated alcohol, followed by washing with Exalin (Merck) for 1 min, and rinsed thoroughly with double-distilled water. Later, these buds were treated with 0.1 per cent solution of mercuric chloride for 10-15 min. The sterilized buds were rinsed three times with autoclaved- distilled water before transferring them to liquid Murashige and Skoog's (1962) medium (MS) supplemented with 100 mg l^{-1} myo-inositol (Sigma), 30 g l^{-1} sucrose (Hi Media) and 1–10 mg l^{-1} BAP (Sigma). The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 15 min. In all cases, the pH of the media was adjusted by using 1N NaOH and 1N HCl.

Shoot multiplication

The proliferated axillary shoots were excised and cut into 3-5 shoot clusters and cultured in liquid MS medium supplemented with different concentrations of BAP and Kn

either singly or in combination of Kn with the optimum concentration of 2 mg l^{-1} BAP. After first shoot multiplication, the shoot cultures were cut into a group of 3 shoot clusters called as propagules and regularly subcultured at 3-4 weeks intervals and maintained in the liquid MS medium variously supplemented with cytokinins (BAP and Kn). The shoots in liquid cultures were supported initially by using filter paper bridges but discarded later when the shoots recovered. The shoots were transferred to fresh medium whenever the medium turned brown and shoots become yellow in colour. The number of propagules cultured and number of propagules derived at the end of subculture is regarded as the rate of multiplication.

***In vitro* rooting**

The shoots were cut into propagules of 3-5 shoots and were transferred to different strengths of MS medium and were variously supplemented with IAA, IBA and/or coumarin. Rooting percentage, rooting per propagules and average number of days for rooting were recorded.

Statistical analysis

All computations were made using SPSS software package version 12. The statistical significance was calculated by one-way ANOVA followed by Tukey's comparision tests at the 5 per cent level of significance.

RESULTS

Culture initiation

The sterilization procedures adopted helped to achieve 80 per cent aseptic cultures from buds. Nodal segments rubbed with cotton dipped in dehydrated alcohol without cleaning with running tap water before treatment with 0.1 per cent mercuric chloride solution reduced microbial contaminants significantly. On basal medium, the frequency of bud-break was very low. The early bud-break was observed within 8-10 days on 2-5 mg l^{-1} with 1-3 shoots per explant (Fig.1). At high BAP concentration 6-10 mg l^{-1}, the number of shoots per explant increased to 3-7 shoots per explant (Fig. 2a).

Axillary shoot proliferation

On transfer of axillary shoots to MS liquid medium with BAP, continuous and rapid proliferation took place. Different concentration of 1-3 mg l^{-1} in liquid MS medium showed distinct shoot proliferation yielding 5-10 shoots in 2-3 weeks, which on sub-culture multiplied rapidly (Fig. 2b). High concentration of 5-7 mg l^{-1} BAP in MS medium induced thin, leaf-like shoots, where many of them did not develop further. Browning was obtained at the cut ends of explants and also in the medium, which was overcome by quick transfer to fresh medium.

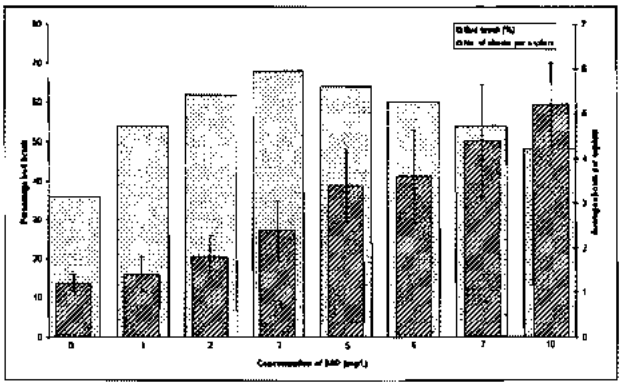

Fig. 1. Effect of BAP supplemented MS medium on bud-break frequency (%) and number of shoots produced per explants after two subcultured cycles from nodal explants of *M. bambusoides*. Bars denote standard error of means.

Shoot multiplication

The excised proliferated axillary shoots were multiplied on MS medium treated with different levels of BAP. The significant multiplication rate was obtained at 3-5 mg l^{-1} BAP. Therefore, 3 mg l^{-1} BAP was selected as the optimum level for continuous shoot multiplication. Following this, other cytokinins such as Kn were used either alone or in conjunction with the optimum concentration of BAP. In all the individual Kn treatments, the shoot multiplication rate was significantly reduced as compared to BAP supplemented medium (Table 1). Of all the BAP and Kn treatments, MS + 2 mg

Table 1. Influence of BAP, Kn and combination of Kn with optimum BAP concentration in MS medium on shoot multiplication after fourth subcultured cycles of *M. bambusoides*

Hormonal treatment (mg l^{-1})	Average number of shoots produced*	Multiplication rate*	Average shoot length*
1 BAP	8.50 ± 1.80^{a}	2.67 ± 0.61^{a}	3.88 ± 0.29^{a}
2 BAP	14.17 ± 1.96^{ab}	4.50 ± 0.67^{ab}	3.62 ± 0.20^{a}
3 BAP	17.00 ± 0.4^{b}	5.33 ± 0.21^{b}	3.58 ± 0.21^{a}
5 BAP	16.67 ± 0.95^{b}	5.33 ± 0.33^{b}	3.54 ± 0.21^{a}
6 BAP	12.00 ± 2.74^{ab}	3.67 ± 0.76^{ab}	3.44 ± 0.33^{a}
7 BAP	10.83 ± 0.23^{ab}	3.17 ± 0.83^{ab}	3.17 ± 0.40^{a}
1 Kn	10.00 ± 1.93^{a}	3.00 ± 0.73^{a}	3.42 ± 0.45^{a}
2 Kn	13.67 ± 1.78^{ab}	4.33 ± 0.61^{ab}	3.45 ± 0.44^{a}
3 Kn	14.33 ± 1.54^{ab}	4.33 ± 0.42^{ab}	3.56 ± 0.54^{a}
5 Kn	15.83 ± 1.28^{ab}	4.83 ± 0.48^{ab}	3.66 ± 0.49^{a}
1 Kn + 2 BAP	17.17 ± 0.87^{b}	5.50 ± 0.34^{b}	3.10 ± 0.32^{a}
2 Kn + 2 BAP	18.17 ± 0.31^{b}	5.83 ± 0.17^{b}	3.60 ± 0.52^{a}
3 Kn + 2 BAP	15.83 ± 1.14^{ab}	4.83 ± 0.48^{ab}	3.37 ± 0.48^{a}
5 Kn + 2 BAP	12.67 ± 1.89^{ab}	4.00 ± 0.68^{ab}	3.00 ± 0.37^{a}

*Mean of 10 replicates ± S.E.
Mean followed by the same letter did not differ at $P< 0.05$

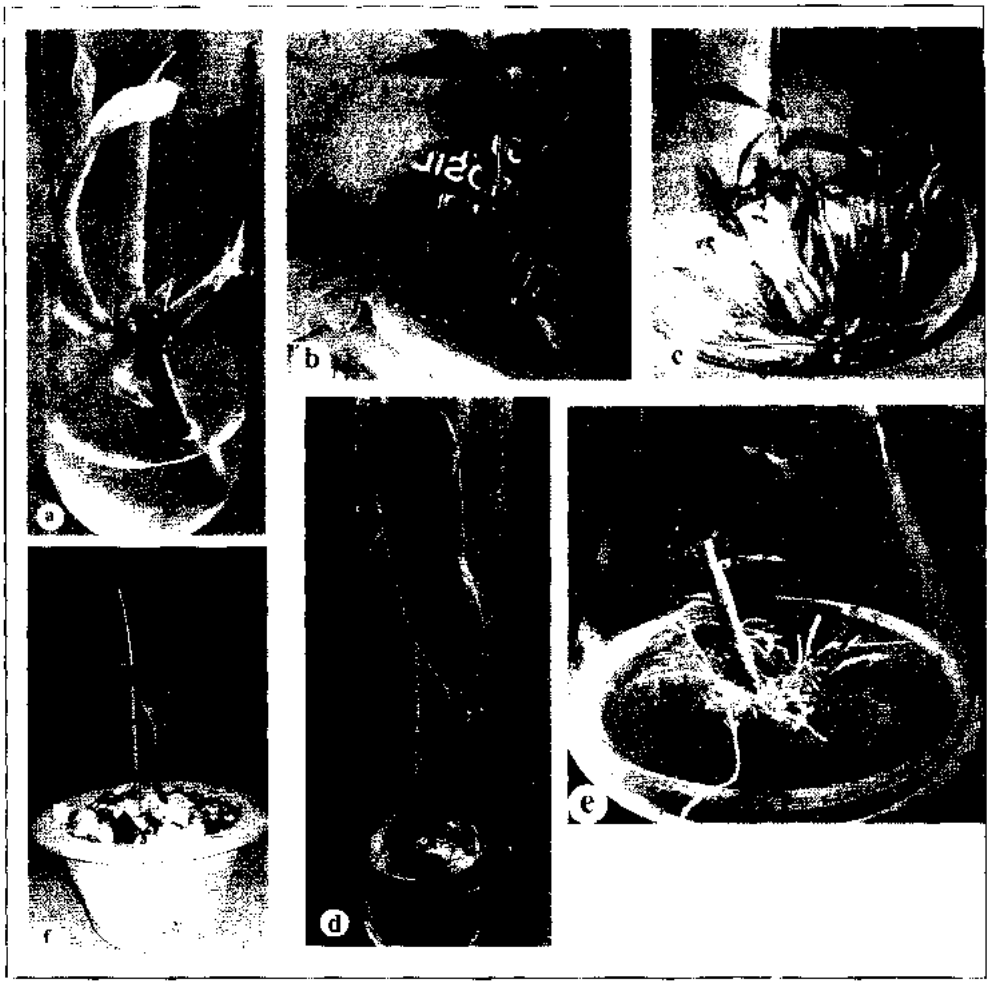

Fig. 2. Micropropagation in *M. bambusoides*. a: Axillary shoots sprouting from a single node in liquid medium; b: axillary shoot proliferation in liquid medium; c: multiplication in MS + 2 mg l^{-1} Kn + 2 mg L^{-1} BAP; d: root induction in ½ MS + 3 mg l^{-1} IBA + 10 mg l^{-1} coumarin in liquid medium; e: rooting in ½ MS + 3 mg l^{-1} IBA + 10 mg l^{-1} coumarin + 0.05 mg l^{-1} BAP in semi-solid medium; f: acclimatization of tissue culture raised plantlets.

l^{-1} BAP + 2 mg l^{-1} Kn produced maximum shoot rate (Fig. 2c). The average shoot length obtained was insignificant at different concentrations of hormone which were tested.

In vitro rooting

The *in vitro* shoots were excised into propagules of 3-5 shoots and were transferred to root induction medium supplemented with IAA, IBA and coumarin singly or in combination with optimum coumarin concentration. Of all the basal media used, MS medium proved to be the best for *in vitro* rooting as compared to WPM medium. The

effect of different strengths of MS salts on the medium was studied at combination of 3 mg l^{-1} IBA and 10 mg l^{-1} coumarin for *in vitro* rooting. At ½ MS strength, more number of roots developed from the cultured propagules. On further increasing the MS strength (*i.e.* ¾ and full MS), a reduced root development as well as rooting response was noticed (Fig. 3). At the optimal concentration of 3 mg l^{-1} IAA and 3 mg l^{-1} IBA, the rooting obtained was 30 per cent and 50 per cent respectively in 30 days (Table 2). The rooting and its percentage significantly increased when 10 mg l^{-1} coumarin was added to the optimum concentration of IAA and IBA. No rooting took place when coumarin was supplemented alone in MS medium. Incorporation of 10 mg l^{-1} coumarin with 3 mg l^{-1} IBA increased the rooting to 75 per cent producing 2.8 roots (Fig. 2d). The incorporation of 0.05 mg l^{-1} BAP along with 3 mg l^{-1} IBA and 10 mg l^{-1} coumarin enhanced the rooting to 82 per cent in 37 mean days, helping development of a healthy root and shoot system. After root induction took place, the rooted shoots were transferred to ½ strength MS medium supplemented with 0.05 mg l^{-1} BAP gelled with 0.2 per cent gelrite which was effective to prevent from inhibitory effect of auxin and vitrification of leaves (Fig. 2e).

Transplantation

In vitro rooted shoots were transferred to culture bottles filled with soil: sand (2:1 v/v) mixture. The transplanted plants were hardened for 20-25 days in the culture room under relative humidity (RH) of 80-90 per cent maintained at 30±2°C. The plants were fed with ½ strength MS medium. Later, the hardened plants were transferred to earthen pots containing sand:soil: farm yard manure (FYM) in 1:1:1 under shade house for acclimatization (Fig.2f). After one month, the plants were planted out in the field during June-October.

Table 2. Effect of treatments on rooting of *M. bambusoides* in half-strength MS medium

Treatments (mg l^{-1})	Rooting percentage	Mean number*	Mean days*
3 IAA	30	1.40 ± 0.68^{a}	30.00 ± 2.22^{a}
3 IBA	55	1.80 ± 0.92^{a}	30.00 ± 2.07^{a}
10 Coumarin	0	0	0
3 IAA + 10 Coumarin +0.05 BAP	68.33	2.40 ± 0.68^{ab}	42.50 ± 2.51^{b}
3IBA + 10 Coumarin + 0.05BAP	75	2.80 ± 0.58^{ab}	40.83 ± 2.71^{ab}
3 IAA + 10 Coumarin +0.05 BAP	80	4.00 ± 0.89^{ab}	35.00 ± 2.58^{ab}
3IBA + 10 Coumarin + 0.05BAP	82	4.80 ± 0.58^{b}	37.00 ± 3.42^{ab}

*Mean of 10 replicates ± S.E.
Mean followed by the same letter did not differ at $P< 0.05$

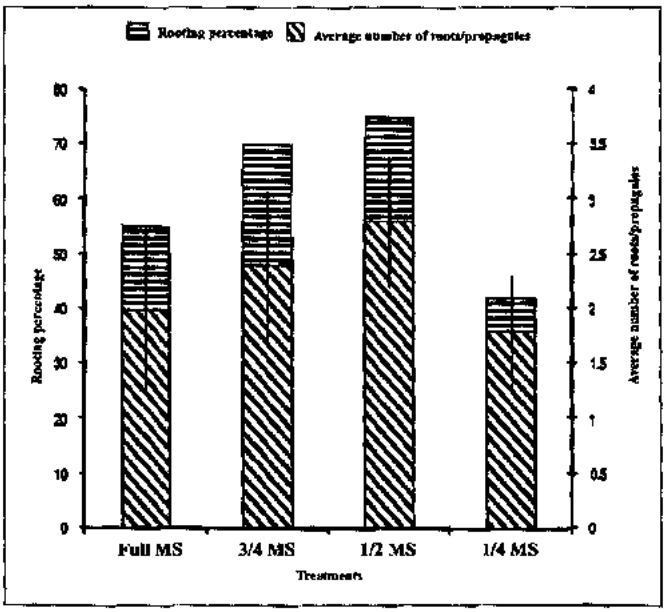

Fig. 3. Effect of different strength of MS supplemented with 3 mg l^{-1} IBA and 10 mg l^{-1} coumarin on rooting percentage and average root number of *M. bambusoides*.

DISCUSSION

The 'jhum cultivation' practice of agriculture, which is prevalent in hilly regions of Manipur, has caused extensive damage to the natural forests of bamboos. There is gradual reduction of fuel wood in the State during the last two decades. The revamping of these denuded forests is extremely difficult due to the lack of planting material. In the present scenario, the propagation of bamboo by tissue culture offers an effective *ex situ* conservation strategy for ecorestoration since other conventional vegetative propagations are associated with a number of problems. The major problems encountered during micropropagation of bamboo using mature plant are culture initiation and rooting. In the present investigation, nodes from secondary branches of *M. bambusoides* collected during the onset of monsoon showed maximum *in vitro* bud-break. Similar findings were reported earlier indicating that culture initiation is affected by growth seasons in *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993) and *D. giganteus* (Ramanayake and Yakandawala, 1997). In the present study, browning was observed at the cut ends of explants and matured nodes turned brown even after transferred to fresh medium. Such browning of explants and proliferated axillary shoots was earlier reported in *D. strictus* (Nadgir *et al.*, 1984; Chaturvedi *et al.*, 1993). The browning obtained in the present experiments was overcome by quick transfer into the fresh medium. The use of activated charcoal and antioxidant solutions were not required in the present case, though its importance has been reported in *Psidium guajava* (Amin and Jaiswal, 1987) and *Maytenus emarginata* (Rathore *et al.*, 1992). Many reports on tissue culture propagation of bamboos such as *D. strictus*

(Chowdury *et al.*, 2004), *B. vulgaris* (Hirimburegama and Gamage, 1995; Ramanayake *et al.*, 2006), used cytokinins in common for bud-breaks and axillary shoot proliferation. Incorporation of 1-10 mg l^{-1} BAP into the medium improved the incidence of bud-break and promoted axillary bud proliferation in *M. bambusoides*. A high rate of shoot multiplication was obtained at optimal concentration of 3 mg l^{-1} BAP. But in earlier reports, high levels of BAP were used for *in vitro* micropropagation of 54 species from 15 genera of bamboos (Prutpongse and Gavinlertvatana, 1992), *D. giganteus* (Ramanayake and Yakandawala, 1997) and *B. bambos* (Arya and Sharma, 1998). In the present investigation, a higher level of BAP brought about stunting of shoots with repeated subcultures. Comparatively, the use of Kn did not produce significant shoot multiplication in agreement with the report of Bhati *et al.* (1992) that BAP for shoot multiplication is followed by kinetin. However, the combined effect of BAP and Kn significantly enhanced the shoot multiplication rate. This stimulatory effect of BAP and Kn on shoot multiplication have also been reported in *D. strictus* (Ravikumar *et al.*, 1998).

In *D. giganteus* and *D. strictus* incorporation of growth regulators like coconut milk and casein hydrolysate was found to be mandatory for shoot proliferation (Ramanayake and Yakandawala, 1997; Ravikumar *et al.*, 1998). However, no such requirement was felt necessary in the present study. The present study reveals that highest rate of shoot multiplication and best growth is obtained in liquid than in semi-solid medium. Similar to this report, incorporation of agar resulted in significant decline in the rate of shoot multiplication in *D. strictus* (Nadgir *et al.*, 1984) and *B. tulda* (Saxena, 1990).

Few reports are available for successful achievement of rooting percentage from adult bamboo species. Bamboos show varying requirements for root induction. Axillary shoots of *D. asper* and *B. bambos* rooted well in the presence of NAA or IBA in the rooting medium (Arya and Sharma, 1998; Arya *et al.*, 1999). In *B. vulgaris*, rooting was enhanced when *in vitro* shoots from adult field culms were pretreated with thidiazuron before placing in the root induction medium (Ramanayake *et al.*, 2006). In the present study, the problem of root induction was overcome by applying coumarin in addition with IAA and IBA. The action of phenolic compounds like coumarin in root promotion could be, at least partly, in protecting the root-inducing, naturally occurring auxin-indoleacetic acid from destruction by indoleacetic acid oxidase (Basu *et al.*, 1969). Kamel *et al.* (2004) suggested that coumarin and IBA may act synergistically in increasing the endogenous free IAA level during the induction phase of rooting to initiate more roots. From earlier reports, it is known that the use of phenolics compounds stimulate *in vitro* rooting in many bamboo species from nodal explants of mature plant (Chaturvedi *et al.*, 1993; Ramanayake and Yakandawala, 1997). However, in comparison to earlier reports, the rooting percentage obtained was high about 82 per cent following the method adopted in the present investigation. The present experiment reveals that addition of low level of BAP improves the quality of shoots which become green and healthy. Chaturvedi *et al.* (1993) observed that

auxins caused inhibitory effects to root for further growth and development. In the present study, the inhibitory effect of auxin was overcome by transferring the plantlets into ½ strength MS medium containing low level of BAP. At the same time, acclimatization of *M. bambusoides* plantlets has been achieved easily with 70-80 per cent survival by way of simply keeping the plants in shade house after transferring into pot containing sand:soil:FYM in 1:1:1 ratio and watering twice a day.

To our knowledge, this is the first report in which a detailed protocol has been successfully developed for rapid micropropagation *of M. bambusoides* from nodal segments of adult, field grown clump achieving high percentage of rooting (82%) and establishment of plants in soil.

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