Wudpecker Journal of Agricultural Research Vol. 1(10), pp. 424 - 428, November 2012
Available online at http://www.wudpeckerresearchjournals.org
2012 Wudpecker Research Journals
ISSN 2315-7259

Full Length Research Paper

Evaluation of rooting response of stem cuttings and in *vitro* micro-cuttings of bottlebrush tree (*Callistemon viminalis*) for commercial mass propagation

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Accepted 13 November 2012

Poor rooting response is a major obstacle in micropropagation as well as conventional plant propagation. Researchers and nurserymen are looking for simple applicable methods to improve root induction in difficult to root species. Bottlebrush (*Callistemon sp.*) is an attractive and popular ornamental shrub with certain rooting difficulty in some species. In the present study, the mass multiplication of bottlebrush through i*n vitro* and *ex vitro* conditions was compared and different concentrations of indole-3-butyric acid (IBA) for rooting of cuttings and micro-cuttings were optimized. Semi-hard wood stem cuttings were treated with IBA at the rate of 0, 2000, 4000 and 6000 mg/l for five seconds. These were then inserted in rooting medium comprising washed sands under misting scheduled for two minutes spray in every 40 minutes. The micro-cuttings were also inoculated on MS (Murashige and Skoog) or ½ MS basal medium supplemented with 0, 2, 4 and 6 mg/l of IBA and the possibility of direct root induction on them were investigated. According to the results, it may be stated that vegetative propagation of *C. viminalis* may be efficiently carried out using semi-hard wood cuttings pretreated with 2000 mg/l IBA under mist system. The *in vitro* inoculation of *C. viminalis* micro-cuttings also may give rise to rooted plantlets but due to tedious steps of sterilization, longer process and higher cost may be discouraged. The cutting procedure described here may be resulted to rapid rooting, bigger rooted plants in a shorter period, much easier technique and shorter acclimation period as compared to micro-cuttings. All these factors may be commercially important.

Key words: *Callistemon viminalis*, rooting, cutting, micro-cutting.

INTRODUCTION

Bottlebrush (*Callistemon viminalis*) is an attractive and popular ornamental shrub of myrtaceae family mostly grown in tropical and sub-tropical regions. The falling branches and red flowers in cylinder spikes are considered as main ornamental objectives of this evergreen shrub (Shokri, 2012). Plants generated from seeds and layering are commonly methods of bottlebrush propagation. However, seedlings may not be true to the type, so sexual propagation method is not reliable (Hartman et al.,, 2002).Vegetative propagation of bottlebrush is also difficult due to relatively difficult to root nature of stem cuttings which demands special hormonal and environmental treatments for root induction(Zarinbal et al., 2005). There are numerous citations on root induction in stem cuttings of difficult to root species facilitated by auxin treatment, bottom heat, wounding and mist system (Dawson and King, 1994, Hartman et al., 2002, Dominik and Gregor, 2007, Shokri, 2012).

The effects of growth regulators (IAA, NAA, IBA at the rate of 2000, 4000 or 6000 mg/l) on softwood cuttings of *C. lanceolatus* were investigated and the highest rooting percentage was achieved by application of 6000 mg/l IBA (Balakrishna and Bhattacharjee, 1991). However, in a Persian citation, IBA at the rate of 4000 mg/l was found to generate highest rooting response in semi hard wood cuttings of *C. viminalis* (Zarinbal et al., 2005).

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Micro-propagation is a massive multiplication *in vitro*, where plants are produced phenotypically and genotypically identical to the original plant from where they derive (Alizadeh, 2007). The *C. viminalis* is a species with a high ornamental and medicinal value that can be multiplied through this technique (Shokri, 2011; Perez and Gradaille, 2011). Currently, more than 30 species of *Callistemon* are described, all with Australian origin and some of them were already propagated under *in vitro* conditions (Perez and Gradaille, 2011). In another attempt, eleven species of myrtaceae family with potential to be used as new ornamental plants were evaluated for their ability to be cultured *in vitro*. The influence of different combinations of growth regulators and basal media were optimized for various species. Furthermore, an increase in root number and an associated decrease in root length were observed as the concentration of auxin (IBA, NAA) was increased (Speer, 1993). *In vitro* culture of bottlebrush tree was also the topic of interest of other researchers (Papafotiou and Skylourakis, 2010; Shokri, 2012). In the present study, it was attempted to establish a protocol for direct root induction in stem cuttings of *C.viminalis* under *in vitro* and glasshouse conditions.

MATERIALS AND METHODS

Glasshouse studies

Semi-hard wood stem cuttings were selected from mid-section of young branches of current season growth. The cuttings were prepared approximately 10-15 cm in size and $^2/_3$ of their bottom portion were defoliated. In order to facilitate rooting the bottom of each cutting was manually wounded, thus some vertical scratches were applied using a sharp scalpel at the distal end of each cutting. The stem cuttings were treated with IBA at the rate of 0, 2000, 4000 and 6000 mg/l for five seconds (quick dip method). These were then inserted in rooting medium comprising washed sands under mist system. Misting was scheduled for two minutes spray in every 40 minutes to maintain average relative humidity of greenhouse in 70%±5. Air temperature and light intensity fluctuation on the bench surface of greenhouse were 25-35°C and 11,000-14,000 lux during mid-day, respectively. In order to prevent fungal infection a general recommended fungicide (Benomyl 0.2% w/v) was also sprayed every 20 days on to the bench surface.

In *vitro* studies

The plant materials used for *in vitro* studies were micro-cuttings of 2-3 cm in size. These were inoculated on MS basal medium supplemented with 0, 2, 4 and 6 mg/l of IBA and the possibility of direct root induction on them were investigated. These were also containing 3-4 nodes and their basal leaves were removed. The explants first were washed thoroughly with tap water and then rewashed with soap and water, then pre-treated with a fungicide solution of benomyl (2 g/l for one hour). The samples were transferred to a laminar hood where dipped in an ethanol solution (60% for 20 seconds). After washing with autoclaved distilled water, cuttings were dipped in the 60% sodium hypochlorite solution for 30 minutes. Finally micro-cuttings were rinsed 5 times using autoclaved distilled water. Plant materials then were inoculated in the test tubes containing basal MS and half-strength MS medium supplemented with Sucrose (30 g/l), myoinositol (100 mg/l), glycine (20 mg/l) and above mentioned IBA concentrations. The pH of medium was adjusted to 5.7-5.8. All the media used were in liquid form and in order to keep micro-cuttings normally, a paper bridge were installed prior to autoclaving.

Data collection and analysis

Forty five days after planting the rooted cuttings were removed from greenhouse beds. However, in case of *in vitro* inoculated micro-cuttings their rooting response was recorded 6o days after inoculation. Rooting percentage, number of roots per cutting, root length and number of new emerged shoots were recorded. Rooted cuttings were transferred to10 cm clay pots containing a potting mixture of soil: cocopeat (2:1). The rooted micro-cuttings also were transferred to jam bottles filled with a sterilized mixture of perlite: sand (1:1) presoaked in ½ MS mineral salts. The polyethylene caps were loosened gradually and finally removed by the end of 3rd week.

The experiment was conducted as completely randomized design with 25 replications in glasshouse experiment and at least 10 replications with respect to *in vitro* studies. The data were analyzed using SAS software.

RESULTS

Glasshouse studies

There was significant difference in rooting response of bottlebrush cuttings with regard to various concentrations of IBA. Despite to some previous citations that reported the bottlebrush as a hard-to-root species (Hartman and et al, 2002); 100 percent rooting was observed in cuttings treated with IBA (2000, 4000 mg/l) as well as control, non-treated plantlets. However, they were different with regard to number of roots per each cutting (Table 1). The rooting percentage was declined (66.67 %) in cuttings treated with 6000 mg/l IBA. Though some variations may be observed between two IBA levels (2000 and 4000 mg/l) but their effects on all measured parameters were found to be not significant. Generated roots in cuttings treated with IBA, had more secondary roots than control (data not shown).

In *vitro* studies

A considerable number of inoculated micro-cuttings were turned yellow to brown color by the first week of culture without any sign of contamination. These symptoms were observed in both leaves and stems and in high degree of severity these were considered as dead. The dead samples were generally more when full strength MS medium was utilized as compared to ½ MS. The contaminated cultures and those considered as dead samples were removed from experiment and data collection. In the other hand, some micro-cuttings were looked alive with the production of roots and new shoots.

Table 1. The effects of various IBA concentrations on rooting response of semi-hardwood cuttings of *Callistemon viminalis* under misting and glasshouse conditions.

	IBA (mg/l)			
Rooting response	**Control**	**2000**	**4000**	**6000**
Rooting percentage (%)	100.00 **a***	100.00 **a**	100.00 **a**	66.67 **b**
Number of emerged roots per cutting	10.17 **b**	19.23 **a**	20.83 **a**	18.20 **a**
Number of new shoots per cutting	0.87 **a**	0.90 **a**	0.73 **a**	0.40 **b**
Average root length per cutting (cm)	7.27 **a**	6.21 **a**	5.97 **a**	3.88 **b**

* Means in the same row followed by different letters are significantly different at P < 0.05 using Duncan'sTest.

The rooting response of micro-cuttings under *in vitro* condition is comparable to the inserted cuttings under glasshouse conditions (Table 2). The results demonstrated that direct root induction was occurred in all media supplemented with IBA as well as control treatment. However, highest rooting (55.56%) and higher number of roots per micro-cutting (1.89) were recorded in media supplemented with IBA 4.0 mg/l. The first root initials were also observed in media containing IBA 6.0 mg/l but it was not significant as compared to 2.0 and 4.0 mg/l IBA. The influence of two basal media (half and full strength MS) was also evaluated on rooting response of micro-cuttings and it was found that the full strength MS inoculated samples developed higher rooting percentage as compared to half strength one (Figure. 2). The effect of basal medium on other rooting parameters was not statistically significant (data not shown).

DISCUSSION

Poor adventitious root formation is a major obstacle in micro-propagation and in conventional propagation (De Klerk, 2002). The role of auxin treatment in root induction has been proved long time ago (Howard, 1973; Hartman et al., 2002) and it has been reported that auxin exerts primary role in root formation by its involvement in successive and interdependent phases (Bellamine et al., 1998). Induction of root in the cuttings depends to the presence of endogenous auxin inside the plant tissues and its synergistic effect with exogenous ones leads to the synthesis of ribonucleic acid and as a result induces root primordial (Hartman et al, 2002).

The conventional propagation methods of ornamental bottlebrush plant are considered to be seed and layer. Layering is a difficult vegetative propagation method and does not always succeed (Perez and Gradaille, 2011). The great interest of this species for being used in parks, avenues and gardens makes necessary to research about different propagation methods that be faster and safer. The study of the rooting response of bottlebrush semi-hard wood cuttings was undertaken as a preliminary experiment in our laboratory. The cuttings (15 to 20 cm in length) were collected from stock plants in different seasons and inserted in rooting media under outdoor conditions. These were received irrigation thrice a day; however the rooting percentage was considerably low and poor success was achieved (data not shown). In order to improve rooting of cuttings a mist system was designed (Figure 1). The high frequency of root induction was observed even in control samples without any hormonal treatment (Table 1). These results confirmed the usefulness of mist system as compared to our preliminary pilot experiment. The effectiveness of misting in propagation of many horticultural species was already reported in previous studies (Balakrishna and Bhattacharjee, 1991; Swetha, 2005; Dominik and Gregor, 2007; Gautam et al., 2010). Besides misting, auxin also plays the essential role in rooting response as its activity may lead to hydrolysis and transport of carbohydrates and nitrogenous materials in the base of cuttings which itself leads to cell division and elongation in this area. Another possible reason may be due to the formation of initial roots and consumption of more stored nutrients in the treated cuttings under the mist system (Ajaykumar, 2007).

According to the results (Table 1), as there is no statistical difference between two IBA levels (2000, 4000 mg/l) with regard to measured rooting parameters, the lower concentration is proposed as optimum level for hormonal treatment. Such level enhances rooting response through development of greater number of roots per cutting. Furthermore, new emerged shoots in cuttings received this treatment, were recorded to be higher than control. Such conditions may improve the subsequent growth and establishment of newly rooted plantlet as already pointed out by Dawson and King (1994). Since the formation of new leaves and shoots on original cuttings were preceded root formation, it can be argued that development of new shoots and production of young leaves may enhance photosynthesis and assimilation of carbohydrates and as a result increasing in generation of more new roots (Shokri, 2012).

Application of IBA at the rate of 6000 mg/l was found to decrease rooting response of bottlebrush cuttings. The rooting percentage, root length and production of new shoots also were significantly decreased in cuttings treated with high concentration of IBA. Negative effect of high hormonal dose and its toxic effect were already reported in propagation of Phalsa (*Grewia subinaequalis*)

Table 2. The effects of various IBA concentrations on rooting response of micro-cuttings of *Callistemon viminalis* under *in vitro* conditions.

	IBA (mg/l)			
Rooting response	**Control**	**2.0**	**4.0**	**6.0**
Rooting percentage (%)	16.67 **b***	50.00 **a**	55.56 **a**	29.28 **ab**
Number of roots per micro-cutting	0.17 **b**	0.67 **b**	1.89 **a**	0.50 **b**
Number of days to root emergence	54.00 **a**	41.10 **b**	48.03 **b**	39.50 **b**

* Means in the same row followed by different letters are significantly different at P < 0.05 using Duncan's Test

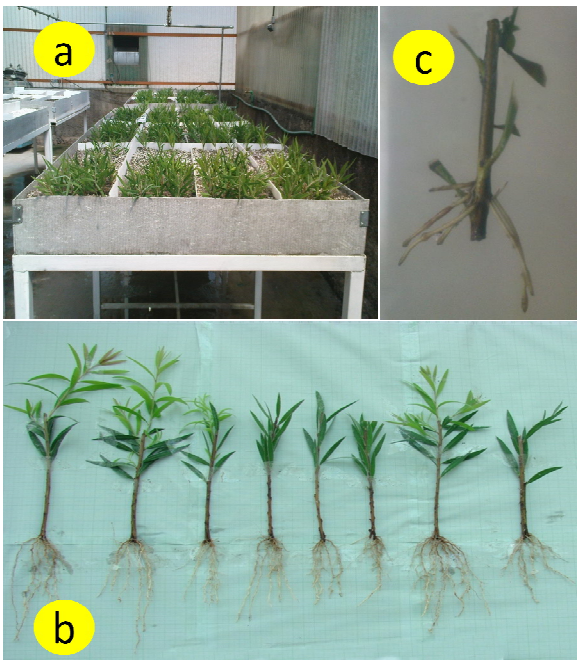

Figure 1. The bottlebrush semi-hard wood cuttings inserted in glasshouse beds under mist system (a); Rooted cuttings lifted up from glasshouse beds 40 days after planting (b); A rooted micro-cutting 45 days after inoculation in MS medium supplemented with IBA 6 mg/l (c).

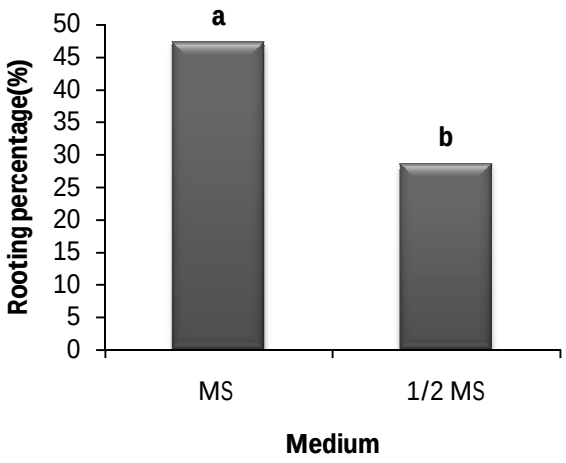

Figure 2. Influence of full and half strength MS basal media on rooting percentage of micro-cuttings of *C. viminalis* under *in vitro* conditions.

through cutting. (Ajaykumar, 2007). However, in case of *Callistemon lanceolatus,* which is a difficult to root species of bottlebrush, the highest rooting percentage (80%) was attained in 6000 mg/l IBA (Balakrishna and Bhattacharjee, 1991).

Auxin causes cell enlargement and elongation of the root cells. However, high concentrations of auxin may have negative effect on root elongation (Hartman et al., 2002) as confirmed in our results (Table 1).

Review of the literature revealed that auxin application may increase the number of roots per each individual cutting (Hussein, 2008; Nair *et al,* 2008). There are some data on the enhancement of the respiratory rate and presence of high levels of stored amino acids in the base of the treated cuttings, 24 hours after external application of IBA (Shokri, 2012).The results of the present study were similar to those of Sun and Bassuk (1993) who reported that the number of generated roots per cuttings of rose "Royal T" may be affected by the auxin levels.

In the present study, the possibility of direct root induction in freshly harvested micro-cuttings was evaluated under *in vitro* conditions. Though roots were induced in a number of inoculated micro-cuttings (Table 2), but it seems that the degree of success is not as good as mist propagation under glasshouse beds (Table 1). Most of the rooted micro-cuttings failed to overtake the acclimatization process and all were died as soon as they were transferred to *ex vitro* conditions. Hence, according to the results of the *in vitro* studies, inoculation of micro-cuttings for direct root induction is not recommended. There are numerous reports and reviews on adventitious root formation in micro-cuttings (De Kelerk, 1997; Barcelo, 1999; De Kelerk, 2002; Ansar et al., 2009).

However, they have all used the micro-cuttings procured from *in vitro* materials following successive sub-cultures. In order to develop an efficient protocol for *in vitro* root induction in bottlebrush micro-cuttings, presently a series of media and growth regulators have been selected and their evaluation is under process in our tissue culture

laboratory.

In conclusion, it may be stated that vegetative propagation of *C. viminalis* may be efficiently carried out using semi-hard wood cuttings pretreated with 2000 mg/l IBA under mist system. The *in vitro* inoculation of *C. viminalis* micro-cuttings also may give rise to rooted plantlets but due to tedious steps of sterilization, longer process and higher cost may be discouraged. Moreover, for *in vitro* root induction of micro-cuttings, an efficient micropropagation protocol may be standardized and *in vitro* generated shoots may be evaluated for their rooting response. The cutting procedure described here may be resulted to rapid rooting, bigger rooted plants in a shorter period, much easier technique and shorter acclimation period as compared to micro-cuttings. All these factors may be valuable from commercial point of view.

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