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HIGH FREQUENCY PLANTLET REGENERATION OF FIRE FLAMED BUSH [*WOODFORDIA FRUTICOSA* (LINN.) KURZ.] USING NODAL EXPLANT

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An efficient high frequency *in vitro* regeneration protocol was developed for *Woodfordia fruticosa* (Linn.) Kurz., a wild threatened medicinal ornamental shrub, which is in need to be conserved by tissue culture techniques. The plants were regenerated from young nodal segments. Maximum number of multiple shoots (166.2±3.89) was obtained from nodal segment explants on MS medium supplemented with 0.5 mg/l BAP with 95% shoot regeneration. Shoot elongation was best in media with BAP 0.2 mg/l. Maximum shoot height of 9-11 cms was achieved. The excised shoots were cultured on MS medium with various concentrations and combinations of auxins for rooting. The best response in rooting was observed on half-strength medium supplemented with 1.0 mg/l IBA. Rooted plants were hardened in earthen pots containing sterile sand before transferring to the field. So, the results of the present studies indicate a five fold increase in number of shoots per explant. This is a rapid, reproducible protocol for large scale propagation of this rare, much- used medicinal, beautiful, ornamental, threatened plant species and its ex-situ conservation.

Keywords: Auxins, Regeneration, multiple shoots, Tissue culture, *Woodfordia fruticosa*.

Fire flamed Bush [*Woodfordia fruticosa* (L.) Kurz.], commonly called as Dhavari, Dhatki,etc. is a plant with medicinal properties and belongs to the family Lythraceae. Flowers of this plant are the most effective fermentation agents in ayurvedic medicines (Kroes *et al*. 1990). It is used both internally as well as externally. The dried flowers of this plant are reported to be used for the treatment of hemorrhoids, dysentery, diarrhoea, liver diseases, piles, disorders of mucous membranes, leucorrhoea, menorrhagia, ulcers, wounds, burning sensations, skin diseases, fever, headache, herpes, etc. (Chadha 1976, Anonymous 1978). They are often added to the Ayurvedic Arishtas to cause alcoholic fermentation (Atal *et al*. 1982).

It has been included in the IUCN Red List of Threatened Species under the category Lower Risk Least Concern (Molur *et al.* 1995), therefore measures are needed to conserve it using tissue culture techniques. Over past several years, tissue culture has rapidly evolved as one of the major research tools and is being used in varied fields. It has an important role to play in solving problems related to economically important plant improvement. (Sharma and Patni 2006; Meena *et al*. 2010).

This is the first report of high frequency plantlet regeneration of *Woodfordia fruticosa* (L.) Kurz. on MS medium and is an improvement over the previously reported protocols for rapid micropropagation.

MATERIALS AND METHODS

Plant Materials

The plants were collected from Rajaji National Park, Haridwar, Shivanand Ashram Campus Area, Rishikesh and also from Nall Sandol Herbal Nursery, Jhadol (Udaipur) and voucher specimens deposited in the Rajasthan University's Herbarium (RUBL20635). They were established in the nursery of Department of Botany, University of Rajasthan and used as

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Table 1: Effect of cytokinins on multiple shoot induction from nodal explants of *Woodfordia fruticosa* on MS medium.

Cytokinins/PGRs (mg /l)		Mean number of multiple shoot ± S.D.*	Shooting Response (%)	Shoot with basal callus intensity
BAP	Kn			
0.2	-	139.6 ± 3.64	90	+
0.5	-	**166.2 ± 3.89**	95	+
1.0	-	132.8 ± 2.77	85	++
1.5	-	124 ± 2.73	70	++
2.0	-	100 ± 5.43	80	+++
2.5	-	97.2 ± 5.35	85	++++
-	0.2	69.8 ± 3.56	80	+
-	0.5	80.2 ± 2.86	85	+
-	1.0	68.6 ± 3.57	85	+
-	1.5	65.4 ± 3.36	70	++
-	2.0	58.4 ± 3.64	70	+++
-	2.5	55 ± 3.39	75	++++

Callus intensity : - + = very low callus, ++ = low callus, +++ = moderate callus, ++++ = high callus.

an explant source. The young nodal segments were excised into 1.0-1.5 c.m. long and were washed thoroughly in running tap water for 30 minutes, followed by washes with Tween 20 for 5 minutes. They were then given anti-oxidant treatment by dipping them in an anti-oxidant solution (Citric acid - 100 mg/l, Polyvinyl Pyrrolidone (PVP) 100 mg/l, Ascorbic Acid 50 mg/l) for 1 hour, due to the leaching of phenolic compounds in the media experienced, if transferred without giving anti-oxidant treatment. Then, they were surface disinfected by mercuric chloride solution, 0.1% (w/v) for 1-2 minutes and thoroughly washed with sterile distilled water 5 times in order to remove the mercuric chloride residue. The explants were excised from the cut ends and were aseptically implanted on MS Medium.

Culture Media And Conditions

The surface disinfected nodal segments were cultured on MS Medium (Murashige and

Table 2: Effect of auxins on rooting response of *in vitro* raised shoots of *Woodfordia fruticosa* on ½ MS medium.

Auxins/PGRs (mg/l)		Mean number of roots ± S.D.	Percentage of rooting response (%)	Average root length (c.m.)
IBA	IAA			
0.2	-	23.8 ± 0.83	75	4.24 ± 0.66
0.5	-	29.8 ± 0.83	80	4.22 ± 0.54
1.0	-	**34.4 ± 0.89**	**90**	**6.5 ± 0.41**
1.5	-	30.4 ± 1.51	85	6.4 ± 0.44
-	0.2	6.8 ± 0.83	70	6.28 ± 0.37
-	0.5	11.4 ± 0.89	80	7 ± 0.41
-	1.0	9.2 ± 1.30	75	6.12 ± 0.39
-	1.5	8 ± 1.58	75	5.18 ± 0.25

Skoog 1962) consisting of basal salts and Vitamins with 3% (w/v) sucrose and 0.8 % (w/v) agar. Plant growth regulators at different concentrations were incorporated into the basal media. The pH of the medium was adjusted to 5.8 by 1N NaOH or 1N HCl before autoclaving at 15 psi for 20 minutes. The cultures were incubated at 25 $\pm$ 2 ºC in a culture room under 16 hour photoperiod illuminated by fluorescent light (2000-3000 lux) and 55 $\pm$ 5 % relative humidity.

Multiple Shoot Induction And Multiplication

For shoot induction and shoot multiplication, the young nodal segments were cultured on MS Medium fortified with different concentrations of BAP (0.2-2.5 mg/l) and Kinetin (0.5-2.5 mg/l). After 4 weeks of incubation, well developed multiple shoots were subcultured on fresh MS Medium consisting of optimal concentration of cytokinin for further shoot elongation. 20 explants were used per treatment and all experiments were repeated thrice.

Rooting And Plant Regeneration

The elongated shoots (10-15 cm) were excised into single shoots and subjected to *in vitro* rooting by culturing on half strength MS medium augmented with various concentrations of auxins, like IAA (0.2-1.5 mg/l) and IBA (0.2-1.5 mg/l). Observations were taken at the end of the 4th week.

Rooted plantlets, after sufficient number of roots had formed, were washed in sterilized water to remove culture medium and drenched with a 0.02-0.2% fungicide like Bavistin and then transferred to autoclaved mixture of fine sand and garden soil (1:1 v/v) filled in small earthen pots and watered regularly and were placed at 25 $\pm$ 2 ºC under cool white flourescent light (2000-3000 lux) with a 16-8 hours photoperiod. The plastic cover was opened after 15-17 days and temperature was gradually increased to 28 ± 2 ºC. The fully acclimatized plants were transferred to the green house.

Experiments were set up in completely randomized design. Data were collected at the end of the 4th week from culture initiation. Data were used to obtain mean and standard deviation.

RESULT AND DISCUSSION

Shoot Induction And Multiplication

The nodal segments were inoculated on MS Medium containing different concentrations of BAP (0.2-2.5 mg/l) and Kinetin (0.5-2.0 mg/l), for the induction of multiple shoots. Within 1-2 days of inoculation, the nutrient medium turned brown due to excessive leaching of phenolics from cut ends of the explants. Survival of the explants reduces if polyphenol interference is left unchecked (Krishnan and Seeni 1994). Exudation of phenolic substances have been reported to be season (Das and Mitra 1990) and age (Mascarenhas *et al*. 1987) dependent. But in this case, it occurred throughout the year, irrespective of the age of explant source. This could be effectively checked by treating the explant with an anti-oxidant solution (as described in Section 2) and also by adding ascorbic acid (75 mg/l) to nutrient medium. Purohit and Tak (1992) also reported prevention of phenolic exudation from nodal segments of *Feronia limonia* explanted on nutrient medium containing an absorbent and anti-oxidant.

Multiple shoots developed from nodal segments with the development of callus at the base. All the concentrations of BAP and Kinetin that were tried facilitated shoot bud differentiation. Generally, cytokinin has been utilized to overcome apical dominance of shoots and to enhance branching of lateral buds from leaf axils (Murashige 1974). The direct effect of cytokinin in tissue culture may vary according to the particular compound used, the type of cultures and the plant species from which it was derived (George and Sherrington

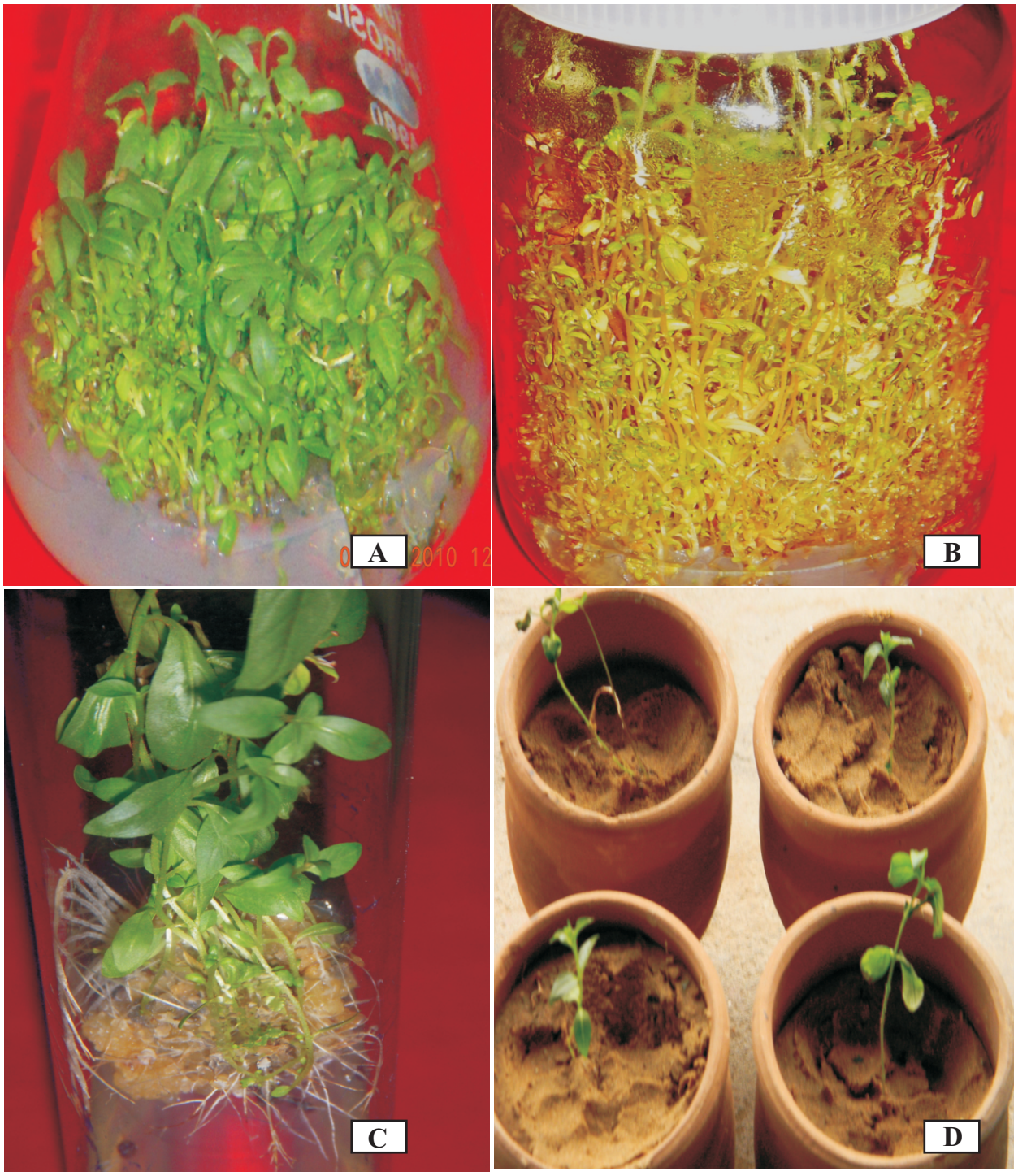

Figure 1. Micropropagation of *Woodfordia fruticosa* from nodal segments. (A) Multiple shoot induction on MS medium supplemented with BAP (0.5 mg/l) (B) Elongation of multiple shoots on MS + 0.2 mg/l BAP (C) Root induction with a network of 25-30 roots on MS + 1.0 mg/l IBA. (D) Hardening of rooted plantlets in earthern pots.

1984). For the induction of multiple shoots in Fire Flamed Bush, cytokinin in the form of BAP was found to be highly essential and efficient. The superiority of BAP over Kinetin for the induction, proliferation and subsequent growth of multiple shoot was also reported by Yadav *et al.* (1990 a) in *Syzygium cumini* ; and Gamboa and Abdelnour (1999) in *Gmelina arborea*. In the present study, maximum number of shoots (166.2±3.89) was obtained from nodal segment explant on MS medium supplemented with 0.5 mg/l BAP (Table 1) with 95% shoot regeneration response.

When BAP concentration was increased from 0.5 to 1.0 mg/l or even more (2.5 mg/l), it reduced the average number of multiple shoots produced. It also resulted in shorter shoots with lesser number of leaves. Production of stunted shoots at higher levels of BAP has also been reported by Yadav *et al.* (1990 b) in mulberry. Shoot induction and multiple shoot development were observed in all concentrations of BAP (0.2-2.5 mg/l). Promotion of shoot multiplication by BAP has been reported in other medicinal plants also like *Holstemma ada-Kodien* (Martin 2002) and *Macadamia tetraphylla* (Mulwa and Bhalla 2000).

When nodal segments were cultured on medium supplemented with various concentrations of Kinetin (0.2-2.5 mg/l), multiple shiny shoots were observed but they were lesser in number than those observed in the medium supplemented with BAP. The highest rate of response obtained with Kinetin was at 0.5 mg/l Kinetin and 80.2 ± 2.86 shoots were developed. So, it was observed that Kinetin as a growth regulator was also effective in multiple shoot induction but not as effective as BAP. BAP induced larger number of shoots as compared to Kinetin. So, the best protocol for the mass propagation of this critically endangered plant species found in the present study for the induction of multiple shoots is the MS medium supplemented with 0.5 mg/l BAP. (Fig. 1) Thus, this medium was designated as "Shoot Proliferation medium or "Shoot Multiplication medium"

Shoot Elongation

Proper shoot elongation was achieved by reducing the concentration of cytokinin in the medium, as shoots failed to elongate on the same medium. So, for elongation of the multiple shoots, BAP at 0.2 mg/l was found to give good results. Thus the medium was designated as "Shoot Elongation Medium". Elongated shoots reached a height of 9-11 cms after transferring to shoot elongation medium.

ROOTING

In vitro raised and elongated multiple shoots were excised into single shoots (9-11 cms) and were rooted on half strength MS medium fortified with various concentrations of auxins (IAA, IBA). Out of different concentrations of IBA (0.2-1.5 mg/l) and IAA (0.2-1.5 mg/l) tested, 1.0 mg/l IBA proved to be the most suitable for root induction with a network of 30-35 roots per shoot and the average root length being 6.5 $\pm$ 0.41 cm (Table 2). Similar effects of IBA were also observed in *Pluchea lanceolata* (Arya *et al*. 2008), and *Centella asiatica* (George *et al*. 2004).

When the shoots were cultured on medium supplemented with various concentrations of IAA, 0.5 mg/l IAA produced thin and long roots which were approximately 10-12 in number. On full strength medium, more callusing was observed in the rooting cultures. So, half strength medium was found to be the best for rooting.. The combination of IBA and IAA was also tried to induce rooting response but it resulted in only lesser number of short rootlets with more of callusing. So, the best response to *in vitro* rooting was observed when the multiple shoots were kept for rooting on half strength medium with 1.0 mg/l Indole Butyric Acid (Table 2). IBA was found more effective in inducing large number of roots as compared to IAA. Development of such strong and lengthy rootlets would be very much helpful for the establishment of plantlets in the

field.

Hardening And Acclimatization

Rooted plantlets were removed from the culture vessels after sufficient number of roots had formed and transferred to small earthen pots containing sterilized mixture of sand and garden soil (1:1 v/v) covered with glass beaker. The fully acclimatized plants were transferred to the green house.The plantlets were successfully transplanted in soil with 70% survivability potential.

It can be concluded that the protocol reported here is an improvement over the previously reported protocols for rapid micropropagation of *Woodfordia fruticosa* (L.) Kurz. It resulted in induction of larger number of both shoots and roots in the *in vitro* cultures on MS medium. So, this protocol is rapid, reproducible and also useful for the conservation and mass propagation of this critically endangered much- used medicinal and ornamental plant and an alternative future source for the bioactive compounds.

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