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Micropropagation via Nodal Explants of *Woodfordia fruticosa* (L.) Kurz.

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ABSTRACT

An efficient protocol for the *in vitro* clonal propagation of *Woodfordia fruticosa* (L.) Kurz using node explants from mature plants was established. Axillary bud proliferation was initiated on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA; 4.40, 8.90, 13.30, 17.70, 22.20 and 26.50 µM) or kinetin (Kn, 4.60, 9.30, 13.90, 18.50, 23.20 and 27.20 µM) alone, or with combinations of BA (2.22, 4.40, 6.62 and 8.90 µM) and Kn (2.32, 4.60, 6.92 and 9.30 µM). MS medium supplemented with BA (17.70 µM) showed the highest regeneration efficiency with 35 ± 1.65 multiple shoots/explant. The shoots that regenerated *in vitro* attained a maximum average length of 4.5 ± 0.25 cm within 5 weeks of culture, 90% of which could root on medium containing half-strength MS salts fortified with 4.90 µM IBA. The regenerated plantlets were established in a greenhouse with 85% survival. ISSR-PCR revealed genetic fidelity among micropropagated plants and the mother plant.

Keywords: cytokinin, fire-flame-bush, ISSR, MS medium, multiple shoots
Abbreviations: BA, 6-benzyladenine; **Kn**, kinetin; **IBA**, indole-3-butyric acid; **ISSR**, inter simple sequence repeat; **PCR**, polymerase chain reaction

INTRODUCTION

Woodfordia fruticosa (L.) Kurz. (*syn*. *Woodfordia floribunda* Salisb), or fire-flame-bush (family – Lathyraceae), is a threatened woody shrub which is in regular demand amongst traditional medicine practitioners of South East Asian countries. In India, this plant is extensively used in Ayurvedic and Unani systems of medicines. Although all parts of the plant possess valuable medicinal properties, there is a high demand for its flowers both in domestic and international markets. It is an ingredient of a preparation that is used to make barren women fertile (Burkill 1966; Dey 1984; Das *et al.* 2007). The red dye extracted from the flowers is extensively used throughout India for dyeing fabrics (Das *et al.* 2007). Many marketed drugs comprise flowers, fruits, leaves and buds mixed with pedicels and thinner twigs of this plant (Das *et al.* 2007). The importance of a tannin, woodfordin I, extracted from *W. fruticosa*, has been documented as a potential lead compound against myelogenous leukemia (Liu *et al*. 2004). The hepatoprotective activity of *W. fruticosa* flowers against carbon tetrachloride-induced hepatotoxicity was determined (Chandan *et al.* 2008). The compound woodfordin C (an inhibitor of DNA topoisomerase II), which exhibits antitumor activity (against human Pancreatic Cancer-1 cell lines), was isolated from methanol extract of *W. fruticosa* (Kuramochi-Motegi *et al.* 1992).

Propagation *via* seed is difficult in this species as seedlings are highly susceptible to wilt caused by *Fusarium* spp. (Bahuguna *et al.* 1988). Moreover, seeds lose their viability within 6 months from collection (Bhagat *et al.* 1992). Vegetative propagation is difficult as this plant is considered to be a hard-to-root species irrespective of season and hormone treatment (Bahuguna *et al.* 1988; Rajesh *et al.* 1993; Raju *et al.* 1994). The threatened status and medicinal importance require the formulation of a reliable, rapid and reproducible micropropagation protocol for *W. fruticosa*. So far only one report has described a protocol for the clonal propagation of *W. fruticosa* using shoot apices (Krishnan and Seeni 1994). The aim of the present study was to establish an *in vitro* regeneration protocol for *W. fruticosa* using nodal explants.

MATERIALS AND METHODS

In vitro propagation

The defoliated twigs of *W. fruticosa* were collected from mature plants growing in Adilabad forest (longitude 77° 47′ to 80° 0′ E, latitude 18° 40′ to 19° 56′ N) during February 2007 and washed thoroughly with running tap water to remove dust and soil particles. The twigs were then placed in 5% Tween-20 (Himedia, Mumbai, India) for 5 min, followed by 3-4 washes in sterile distilled water (SDW). The explants were surface sterilized with an aqueous solution of 0.1% $HgCl_2$ for 4-6 min, followed by 4-5 washes with SDW. Upon the culture of mature nodes on MS medium containing 8.90 µM BA, produced a mean number of shoots (2.3 ± 0.26; here after called as *in vitro*-derived nodal explants). These *in vitro*-derived single node (1.0-1.5 cm) segments proliferation was tested by inoculation into borosilicate culture tubes (150 × 25 cm), each containing 20 ml of MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar, and different concentrations of 6-benzyladenine (BA; 4.40, 8.90, 13.30, 17.70, 22.20 and 26.50 µM), or kinetin (Kn, 4.60, 9.30, 13.90, 18.50, 23.20 and 27.20 µM) alone, or combinations of BA (2.22, 4.40, 6.62 and 8.90 µM) and Kn (2.32, 4.60, 6.92 and 9.30 µM). The explants were cultured for 3 weeks on these regeneration media. For culture establishment and multiplication of shoots, 10 explants (one explants per tube) were used with two replicates each and the experiment was repeated twice. The shoots proliferated without fully formed leaves and that were 1-2 cm long were excised and subcultured for an additional 2

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weeks on the same media for shoot elongation. For micropropagation, the node explants derived from *in vitro* raised plants were subcultured at 2- and 3-week intervals on the above mentioned media. MS medium without plant growth regulators (PGRs) served as the control.

In vitro rooting and acclimatization

The shoots (3.0-4.5 cm) raised *in vitro* (from all PGR treatments above) with 3-4 leaves were isolated and transferred to tubes containing MS medium (full or half-strength) supplemented with different levels of indole-3-butyric acid (IBA; one shoot/IBA treatment; 2.46, 4.90, 7.36 and 9.80 µM). MS medium without IBA served as the rooting control. *In vitro*-rooted shoots (5-weeks-old) were removed from culture tubes, washed thoroughly with water to remove agar and medium constituents. Individual plantlets were potted in plastic jars containing a mixture of sterilized soil and vermiculite (1: 1). The plantlets were covered with polyethylene sheets to minimize the loss of moisture and transferred to a greenhouse under natural light conditions (28°C day, 24°C night, 65% RH). The plants are initially fed with liquid MS (micro- and macronutrients) alternated with sterilized distilled water (SDW) and subsequently supplied with half-strength MS nutrients alternated with tap water and finally with only tap water from the third week.

Culture conditions and statistics

All media pH were adjusted to 5.8 with 0.1 N NaOH before adding agar and autoclaved at 121°C for 15 min. Each culture tube was inoculated with a single explant and plugged with non-absorbent cotton wrapped in two layers of cheese cloth. All cultures were maintained at 25 ± 2°C under white fluorescent light (Philips, India, 65 µE m^{-2} s^{-1}) with a 16-h photoperiod. For culture establishment and multiplication of shoots, 10 node explants were used for each treatment and replicated twice. Experiments were repeated twice. The data pertaining to number of shoots/culture (multiple shoots), mean shoot length (5 weeks after subculture onto regeneration media) and number of adventitious roots forming from *in vitro*-derived shoots was recorded and analyzed statistically using DMRT (Duncan's multiple range test; $P \leq 0.05$) after mean separation using analysis of variance (ANOVA). Data are expressed as the mean ± standard error (SE).

DNA extraction and PCR amplification

Total DNA was extracted from the leaves of the *W. fruticosa* mother plant, from *in vitro* grown shoots derived from the 5th, 8th and 16th subculture as well as from hardened plants according to Doyle and Doyle (1990). Qualitative and quantitative assessment of genomic DNA was carried out by agarose (Seakem LE, Koln, Germany) gel electrophoresis and employed for inter simple sequence repeat (ISSR) analysis. DNA concentration was adjusted to 25 ng/µl with SDW and stored at 4°C. Ten different ISSR primers were tested and on the basis of their suitability. Primers -3((GA) 8T), -4((GA) 8C) and -5((TC) 8C) were selected for a more detailed study. PCR was performed in a reaction volume of 25 µl containing 25 ng DNA, 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 1 U *Taq* DNA polymerase (all PCR reagents Fermentas, Maryland, USA), and 10 pM primer (Kranthi *et al*. 2011) using a thermocycler (Biorad-C1000, Hercules, CA, USA). PCR conditions used for amplification consisted of an initial denaturation step at 94°C for 3 min, 35 cycles of 1 min at 94°C (denaturation), 1 min at 50°C (annealing), and 2 min at 72°C (extension), followed by a final extension of 7 min at 72°C, and a soak temperature of 10°C. The amplified DNA was separated by electrophoresis on 1% agarose gel (35 mV, 3 h). The size of the amplicons was estimated using a 1-kbp ladder (Bridge Biological Products, CA, USA). The gels were visualized and photographed using a gel documentation system (Biorad, USA). All PCR reactions were repeated at least twice to ensure reproducibility.

RESULTS

The efficiency of *in vitro* regeneration varied depending on the source of the explant (i.e. nodal explants derived from mature plants growing in a natural habitat or from *in vitro*-raised shoots). Initially, different concentrations of BA or Kn were tested alone or combinations of BA and Kn to culture node explants derived from a mature plant. Node explants exhibited low regeneration efficiency at all concentrations of BA, Kn, or BA and Kn that were tested. The maximum efficiency (40 and 50%) of axillary bud proliferation was obtained using BA at 8.90 and 13.30 µM, respectively. At these concentrations of BA, 4.0 ± 0.26 to 5.0 ± 1.13 shoots/node with a mean length of 2.0 ± 0.75 to 2.4 ± 0.18 were obtained. The other concentrations of BA, Kn, or BA and Kn, resulted in the regeneration of single shoots with a low efficiency (≤ 20%).

Further studies were conducted using the *in vitro*-derived node explants (8.90 µM BA). The use of BA or Kn, or BA and Kn at all tested concentrations resulted in the induction of multiple shoots from node explants (**Table 1**). As the level of BA or Kn increased, so too did the percentage of explants proliferating shoots; the mean number of shoots increased to between 8.0 ± 0.26 and 35 ± 1.65 multiple shoots from a single nodal explant. A further increase in the concentration of BA (22.20 and 26.50 µM) or Kn (18.50-27.70 µM) resulted in a reduction of the mean number of shoots.

The cytokinin BA (17.70 µM) favored an optimal regeneration response, inducing 35 ± 1.65 multiple shoots with a mean shoot length of 4.5 ± 0.25 cm per node explant (**Fig. 1A**). At 22.20 µM BA, 32 ± 1.13 shoots were obtained. Node explants, when cultured on Kn-supplemented media, also formed multiple shoots; however, the number of regenerated shoots was significantly less than the optimal level of BA (17.70 µM) (**Fig. 1B**). In this set of experiments, the shoots regenerated on medium with either 17.70 or

Table 1 Effect of cytokinins on shoot multiplication from *in vitro*-derived node segments of *Woodfordia fruticosa*.

BA (µM)	Kn (µM)	Responding cultures (%)	Mean no. of shoots/ explant ± S.E.	Mean length of shoot/ explant ± S.E.
4.40		70	8.0 ± 0.26 a	3.0 ± 0.18 b
8.90		70	16 ± 0.23 c	3.5 ± 0.23 c
13.30		75	30 ± 1.00 d	4.0 ± 0.22 d
17.70		85	35 ± 1.65 e	4.5 ± 0.25 d
22.20		85	32 ± 1.13 e	4.5 ± 0.25 d
26.50		75	22 ± 1.34 d	3.2 ± 0.31 c
	4.60	50	10 ± 0.26 b	2.2 ± 0.27 a
	9.30	65	12 ± 0.27 c	3.0 ± 0.18 b
	13.90	70	15 ± 0.18 c	3.2 ± 0.16 c
	18.50	75	10 ± 0.11 b	3.0 ± 0.22 b
	23.20	65	8.0 ± 0.14 a	2.5 ± 0.19 b
	27.70	60	6.0 ± 0.37 a	2.0 ± 0.12 a
2.22	2.32	60	15 ± 0.75 c	2.5 ± 0.14 b
4.40	2.32	75	18 ± 0.53 d	3.5 ± 0.23 c
6.62	2.32	70	17 ± 0.60 d	3.0 ± 0.18 b
8.90	2.32	65	12 ± 0.50 c	3.2 ± 0.24 c
2.22	4.60	70	20 ± 0.51 d	3.8 ± 0.25 c
4.40	4.60	80	28 ± 0.85 d	4.0 ± 0.17 d
6.62	4.60	75	26 ± 0.57 d	3.7 ± 0.21 c
8.90	4.60	65	14 ± 0.51 c	3.2 ± 0.24 c
2.22	6.92	45	14 ± 0.56 c	2.5 ± 0.19 b
4.40	6.92	65	20 ± 0.74 d	3.5 ± 0.25 c
6.62	6.92	60	18 ± 0.58 d	2.7 ± 0.22 b
8.90	6.92	50	12 ± 0.49 c	2.2 ± 0.27 a
2.22	9.30	45	10 ± 0.51 b	3.0 ± 0.29 b
4.40	9.30	55	12 ± 0.34 c	3.2 ± 0.24 c
6.62	9.30	50	14 ± 0.57 c	3.0 ± 0.26 b
8.90	9.30	45	11 ± 0.46 b	2.5 ± 0.29 b

Values are mean of 40 explants ± S.E. In each column means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Fig. 1 Micropropagation of *W. fruticosa* on MS medium. (**A**) Multiple shoot induction from *in vitro*-derived node explants on 17.70 µM BA (3 weeks old); (**B**) induction of multiple shoots on 27.70 µM Kn (3 weeks old); (**C**) well developed shoots (5 weeks old); (**D**) rooting of microshoots on ½ MS fortified with 4.90 µM IBA; (**E**) Hardened plant in greenhouse.

22.20 µM BA attained almost identical mean length (4.5 ± 0.25 cm) (**Fig. 1C**). BA, within the range evaluated (4.40-26.50 µM) was more efficient in terms of shoot proliferation and multiple shoot induction than Kn when used alone or in combination with Kn.

In a separate set of experiments, the *in vitro*-derived node explants (8.90 µM BA) were cultured on MS medium containing BA combined with Kn (**Table 1**). Kn at 4.60 µM with different levels of BA (2.22, 4.40, 6.62 and 8.90 µM) enhanced the induction of multiple shoots by 45-80% (**Table 1**). The most effective combination was 4.40 µM BA and 4.60 µM Kn, resulted in a high frequency of proliferated cultures (80%) and maximum number of shoots/node (28 ± 0.85).

The regenerated shoots (2.0-4.5 cm) could be rooted on half- or full-strength MS medium fortified with different concentrations of IBA. Half-MS containing 4.90 µM IBA was ideal for the development of roots (19.0 ± 0.16) from cut ends of *in vitro*-derived shoots (**Table 2**). On this medium, 90% of shoots rooted within 10-12 days of culture (**Fig. 1D**). Upon transfer to the greenhouse (after 7 weeks) 85% of plantlets survived. The *in vitro* regenerated shoots could also root on full-strength MS medium but with fewer roots (8.0 ± 0.25) and a prolonged culture period (15-17 days). All rooted plantlets performed similarly upon transfer to the greenhouse. No roots developed in the control treatment. The shoots with well developed roots were transferred to pots containing a mixture of sterilized soil and vermiculite (1: 1) and hardened in the greenhouse with an 85% survival rate (**Fig. 1E**).

Table 2 Effect of IBA on adventitious rooting of *in vitro*-regenerated shoots of *Woodfordia fruticosa*.

MS medium	IBA (µM)	% of cultures producing roots	Mean no. of roots/shoot	Mean root length (cm)
Full strength	2.46	55	6.00 ± 0.15 a	1.40 ± 0.20 a
Full strength	4.9	70	8.00 ± 0.20 a	2.00 ± 0.04 d
Full strength	7.36	60	6.10 ± 0.18 a	1.30 ± 0.15 a
Full strength	9.8	50	6.00 ± 0.07 a	1.20 ± 0.30 a
1/2 strength	2.46	75	10.00 ± 0.23 b	1.80 ± 0.07 c
1/2 strength	4.9	90	19.00 ± 0.16 f	3.00 ± 0.13 f
1/2 strength	7.36	80	14.50 ± 0.43 e	2.00 ± 0.08 d
1/2 strength	9.8	60	10.00 ± 0.24 b	1.60 ± 0.05 b

Values are mean of 40 explants ± S.E. In each column means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

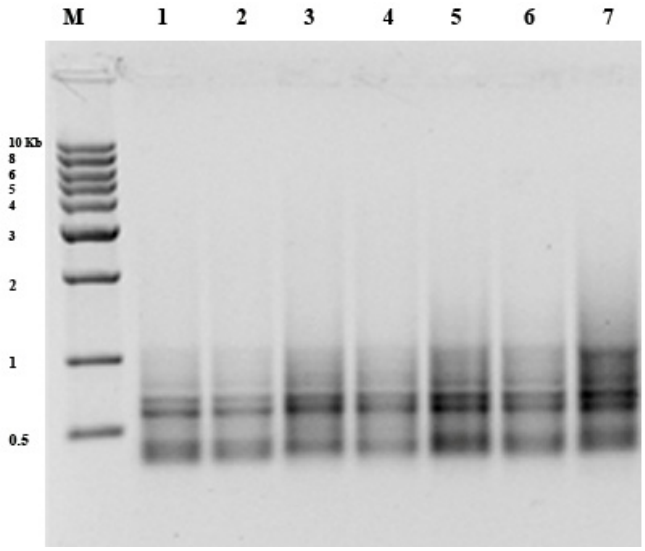

Fig. 2 Establishing clonal fidelity by ISSR analysis. Amplification pattern with primer 3(GA)8T. M, marker; 1, mother plant; 2, hardened plant; 3-7, *in vitro*-grown plants.

Clonal fidelity

Total DNA from *in vitro*-grown shoots of the 5th, 8th and 16th subculture, hardened plants and the mother plant were subjected to ISSR analysis. No polymorphic bands were observed in the mother plant and the *in vitro* raised progeny (**Fig. 2**). Visual analysis of *W. fruticosa* plants also did not reveal any morphological variations. These results confirm that *W. fruticosa* plants obtained by the nodal proliferation retained their genetic fidelity for a prolonged period of 10 months (16 sub-cultures) under *in vitro* conditions.

DISCUSSION

MS medium is routinely and widely used in plant tissue culture studies. However, there are no reports available demonstrating the use of MS salts for the *in vitro* propagation of *W. fruticosa*, which in this study was possible for the first time for axillary bud proliferation and clonal propagation. An earlier study used Schenk and Hildebrandt (SH, 1972) medium for micropropagation of this plant (Krishnan and Seeni 1994). Their results indicated that the highest multiplication of *W. fruticosa* (26-35 shoots/shoot tip) on SH culture initiation media with 2.22 µM of BA and 2.60 µM of NAA followed by subculture in 0.88 µM of BA. The shoot multiplication rate was further accelerated by reculturing 0.4-0.6 cm nodal segments of regenerated shoots in media with 4.40 µM BA. Quraishi *et al*. (1997) reported 2.5 ± 1.9 shoots/explant using basal sprouts of *Lagerstroemia parviflora* in MS medium enriched with 2.22 µM BA.

Among the two cytokinins (BA and Kn) tested in the present study, BA induced highest regeneration efficiency (35 ± 1.65 multiple shoots/explant). BA also induced multiple shoots from nodal explants of *Lawsonia inermis* (Kheta Ram and Shekhawat 2011).

The combination of two cytokinins (BA and Kn) also promoted the development of multiple shoots thereby demonstrating the effect of cytokinin combination on multiple shoot induction in *W. fruticosa*. A combination of two or more cytokinins could induce shoots from various explants of *Eclipta alba in vitro* plantlets when placed on MS medium with BA (4.4 µM), Kn (9.2 µM) and 2iP (2.4 µM) (Baskaran and Jayabalan 2005). Geneve (2005) reported adventitious shoot induction and elongation on root and petiole explants of Kentucky coffee tree (*Gymnocladus dioicus* L.) with a combination of 1 µM BA and 0.5 µM thidiazuron (TDZ). Mahender *et al.* (2009) used a combination of 4.40 mM BA and 4.60 mM Kn for efficient *in vitro* regeneration and micropropagation of a medicinal plant, *Momordica tuberosa* Roxb. Niranjan *et al*. (2010) reported most multiple shoots (24/explant) from *Lagerstroemia indica* using shoot tip culture in medium supplemented with BA (8.9 µM) and IBA (2.46 µM) whereas nodal explants formed most multiple shoots (5/explant) with a combination of KN (9.30 µM) and IBA (2.46 µM).

In vitro regenerated *W. fruticosa* plantlets can be multiplied by subculturing at regular 3-week intervals. This mode of multiplication ensured the continuous production of shoots without a decline in growth performance. Furthermore, our findings on the effect of variations in mineral strength for *in vitro* rooting of microshoots emphasizes the importance of evaluating mineral strength as well as hormone requirements for *in vitro* rooting of *W. fruticosa*. Roy *et al.* (2001) employed ½ MS medium with IBA (4.90 µM) for *in vitro* rooting of *Humulus lupulus* L. Similarly, Dhar and Joshi (2005) reported the use of ½ MS medium containing 2.50 µM IBA for *in vitro* rooting of *Saussurea obvallata* (DC.) Edgew. Our results are similar to the above reports in that ½ MS medium with IBA (4.90 µM) could be effectively used to induce *in vitro* roots.

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