ISSN: 0975-833X

*Available online at http://www.journalcra.com*

*International Journal of Current Research*
*Vol. 4, Issue, 10, pp.040-043 October, 2012*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**RESEARCH ARTICLE**

# HIGH FREQUENCY INDUCTION AND REGENERATION OF MULTIPLE SHOOTS FROM NODAL EXPLANTS OF *Justicia wynaadensis* (NEES) T. ANDERS

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**ARTICLE INFO**

***Article History:***
Received 11th July, 2012
Received in revised form
23thAugust, 2012
Accepted 16th September, 2012
Published online 30th October, 2012

***Key words:***
*Acanthaceae, Justicia,* regeneration, micropropagation, nodal explants, medicinal.

**ABSTRACT**

*Justica wynaadensis* (Nees) T. Anders (*Acanthaceae*) is a small branched shrub and finds potential to be used for the treatment of hypercholesterolemia and atherosclerosis. Micropropagation was attempted with nodal explants on Murashige and Skoog (MS) medium supplemented with growth regulators. Maximum shoots were produced *in vitro* on MS medium supplemented with 3 mg/L BA, with an average of 24.92±0.30 shoots/explant. Rooting was induced in the shoot initiation media itself and did not require a separate rooting media. The rooted plantlets were transplanted to pots containing peat, vermiculite and soil mixture (1:1:1), and maintained in the shade house at a survival rate of 83%.



## INTRODUCTION

*Justicia wynaadensis* (Nees) T.Anders, also called locally as Maddu thoppu, is a small branched shrub, belonging to the family *Acanthaceae*. The aqueous extract is used in the preparation of a sweet dish by the natives of Kodagu district, Karnataka state, India, exclusively during the monsoons as it is believed to possess maximum medicinal properties during the season. This plant is reported to be endemic to the regions of Western Ghats (Gamble, 1967) and common in forests of Irpu (Keshava Murthy and Yoganarasimhan, 1990). Subbiah and Norman (2002) in a patent have reported that the plant extract lowers cellular cholesterol and cholesteryl ester concentration and suggested it's potential use as a food supplement for the treatment of hypercholesterolemia and atherosclerosis. Udayan *et al* (2008) has reported the use of this plant by the local tribes, as an external application over rheumatic swellings. The global market for traditional medicines was estimated at US$ 83 billion annually in 2008, with a rate of increase that has been exponential (Robinson and Zhang, 2011). In India, the population of some of the plants of medicinal value is on the decline in the Western Ghat forests due to overexploitation of minor forest products (Sumana and Kaveriappa, 1996). This plant finds immense potential to be used in pharmaceutical preparations and therefore a rapid multiplication of this plant is necessary to prevent it from being endangered as well as to meet the requirements of the pharmaceutical industry. Poor seed viability and germination has limited the plant's natural propagation. Conventionally, this plant is not propagated since the requirement of this plant during monsoons is met with from natural populations. The plant can be propagated from cuttings but is slow; tissue culture can serve as an alternate method for rapid propagation of this plant for enhanced axillary branching, high frequency multiplication, continuous supply throughout the year and germplasm storage. A perusal of literature revealed that there is no published information on *in vitro* regeneration of *Justicia wynaadensis*. Here we report for the first time, a simple and rapid methodology for the regeneration of *Justicia wynaadensis* using nodal explants.

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## MATERIALS AND METHODS

### Plant material and explants preparation

The plant material was collected from Kodagu, Karnataka, India; it was identified and authenticated at NADRI, Bangalore, as *Justicia wynaadensis* (Nees) T. Anders belonging to the family *Acanthaceae.* Nodal segments (about 4cm long) were washed with tap water followed by washing with a dilute solution of Teepol (1%v/v) for 5 min and then placed under free flowing tap water for 30 min; explants were sterilized by immersion in 70% ethanol for 2 min, followed by 8 min immersion with agitation in 0.1% $HgCl_2$ (w/v). Explants were rinsed with streptomycin solution (30mg/L) for 2 min and finally rinsed five times in sterile distilled water to remove all traces of disinfectants. Nodal explants were trimmed into 2cm long segments and placed onto Murashige and Skoog's (MS) medium.

### Culture Media and Conditions

MS basal medium (Murashige and Skoog, 1962) containing 3% (w/v), sucrose and 0.8% (w/v) agar supplemented with different growth regulators alone or in combination was used in all the experiments. The pH of the medium was adjusted to

5.8 with 0.1N NaOH or 0.1N HCl, dispensed into tissue culture bottles (200ml) and autoclaved at 15lbs psi for 20 min. Inoculated explants were incubated at 25±2°C under 16h photo period at 3000 lux provided by white fluorescent tubes.

### Direct Organogenesis

For direct organogenesis, the explants were placed on full strength/half strength MS medium supplemented with 6-benzyladenine (BA), α naphthalene acetic acid (NAA) and kinetin (Kn) in concentrations ranging from 0.1mg/L - 4.0 mg/L either individually or in combinations as well as in phytohormone free media. At 4-wk intervals the induced multiple shoots were sub cultured on the same media to obtain more cultures or incubated further in the same bottle to allow for rooting.

### Rooting and Acclimatization

After 42 days, culture bottles containing regenerated shoots (about 5 cm long) and bearing roots were transferred to the shade house maintained at room temperature. After a week, the rooted plantlets were separated, washed with tap water to remove all traces of media, immersed in 1% bavistin for one minute and transplanted into pots containing a mixture of peat, vermiculite and soil (1:1:1 v/v) covered with a plastic bag to maintain high humidity. The plastic bags were opened when the shoots reached a height of 12 cm and maintained in the shade house for one month and later transferred to the garden. Experiments were conducted in three replicates with 30 explants per replicate and the data are presented with standard deviation.

## RESULTS

### Multiple Shoot Formation

The nodal explants responded when cultured on basal (phytohormone-free) MS medium as well as in the presence of cytokinins benzyladenine (BA) and kinetin (Kn). The emergence of multiple shoots from nodal explants on MS medium supplemented with BA and Kn was observed without an intervening callus phase. However, the concentration of cytokinins significantly affected the percentage of multiple shoots produced per explant. Basal media did not induce multiple shoot production. Shoot buds emerged within 5 days of culture from single nodal explants. Initially two buds emerge and elongate, followed by the formation of multiple shoots within 15-20 days of culture. In our study, BA at 3mg/L initiated high frequency regeneration (91%) and induced maximum multiple shoots (24.92±0.30 shoots/explant) in 45 days compared to Kn (1.9±0.09 shoots/explant), from single nodal explants (Table 1).The number of shoots increased with increased period of culture. BA at concentrations higher than 3mg/L resulted in poor response, reduced number of shoots and stunted growth. Though Kn induced a response from the nodal explants, shoot growth was slow, and the regenerated plants eventually lost it's vigour. Combination of BA and NAA resulted in poorer response, smaller leaves and reduced number of shoots than when BA was used alone (result not shown). Half-strength MS media incorporated with 3mg/L BA also resulted in poorer response and reduced number of shoots when compared to full-strength MS media (Table 2). Half-strength MS media along with 3mg/L BA and 1mg/L NAA produced elongated shoots with small yellowish-green shoots.

**Table 1. Effect of cytokinins on shoot regeneration from nodal explants of *Justica wynaadensis* on MS medium**

| Growth Regulators | Conc. mg/L | Percentage of explants that responded* | Average no. of shoots/explant* |
|---|---|---|---|
| 0.0 | 0.0 | 84±2.0 | 1.08±0.04 |
| BA | 0.1 | 87±3.5 | 2.05±0.04 |
| | 0.2 | 87±3.5 | 2.08±0.01 |
| | 0.5 | 88±1.9 | 3.73±0.12 |
| | 1.0 | 88±1.9 | 11.37±0.12 |
| | 2.0 | 90±1.9 | 15.09±0.19 |
| | 3.0 | 91±1.9 | 24.92±0.30 |
| | 4.0 | 57±3.4 | 1.61±0.02 |
| Kn | 0.1 | 18±3.9 | 1.00±0.00 |
| | 0.2 | 37±3.4 | 1.00±0.00 |
| | 0.5 | 49±3.8 | 1.00±0.00 |
| | 1.0 | 52±3.9 | 1.00±0.00 |
| | 2.0 | 56±2.0 | 1.10±0.07 |
| | 3.0 | 58±1.9 | 1.90±0.09 |
| | 4.0 | 33±3.4 | 1.00±0.00 |

Observations were taken 45 days after culture.
*The values represent the mean (±SD) of three replicates with 30 cultures for each replicate.

**Table 2. Effect of half-strength MS media on shoot regeneration from nodal explants of *Justica wynaadensis***

| Growth Regulators | Percentage of explants that responded* | Average no. of shoots/explant* |
|---|---|---|
| BA:3mg/L | 88±1.9 | 7.7±0.25 |
| BA:3mg/L +NAA:1mg/L | 87±3.5 | 10.67±0.41 |

Observations were taken 45 days after culture.
*The values represent the mean (±SD) of three replicates with 30 cultures for each replicate.

The clusters of *in vitro* regenerated shoots when separated and sub cultured on MS+3mg/L BA again produced multiple shoots. In our study, the same media served as initiation, elongation and multiplication media.

### Rooting and Transplantation

Rhizogenesis was observed in the shoot initiation media itself, when nodal explants were cultured on full-strength MS / half-strength MS supplemented with/without BA. Roots were not induced during culture initiation, but emerged after a period of shoot formation and shoot multiplication. 6-8 roots were observed within 45 days on shoot initiation media. MS media supplemented with NAA also induced vigorous root formation, but produced smaller leaves on shoots. Thus, in our study a separate rooting media was not required. Nodal segments grown on MS medium supplemented with varying concentrations of Kn (0.1- 4.0 mg/L) did not induce rhizogenesis. The rooted plants were transferred to pots containing sterilized peat, vermiculite and garden soil mixture; 83% of the potted plants established into healthy plants and flowered during the natural flowering period (Jan- Mar).

## DISCUSSION

The *in vitro* propagation of *Justicia wynaadensis*, an endemic plant of the Western Ghats is reported here for the first time. *In vitro* direct organogenesis from node has been reported from other medicinal plants including *Acanthacean* members,

such as *Beloperone plumbaginifolia* (Shameer *et al.*, 2009), *Justicia gendarussa* (Thomas and Yoichiro, 2010), *Stachytarpheta jamaicensis* (Rajender *et al.,* 2012), *Mentha piperita* (Ghanti *et al.*, 2004) and *Adhatoda vasica* (Bimal and Shahnawaz, 2012). In our study, high frequency regeneration(91%) and multiple shoot formation from single nodal explants (24.92 ± 0.30 shoots/explant) was obtained on MS media supplemented with 3mg/L BA.


**Fig.1. *In vitro* regeneration of *Justicia wynaadensis* (Nees) T. Anders**
**A – Nodal explants grown on phytohormone-free MS medium; B – Multiple shoots formed after 4 weeks on MS medium containing 3.0mg/L BA; rhizogenesis also seen; C - Multiple shoots after 45 days; D - Hardening**

A similar study on *Biophytum sensitivum* (Shivanna *et al*., 2009) with 3mg/L BA induced an average of 8.33 shoots with inflorescence tip explants, not with stem explants. In *Andrographis lineate* (Deepa *et al*., 2011) 15 shoots per nodal explant was reported with a concentration of 1.5mg/L BA and 30mg/L adenine sulphate. MS supplemted with 5mg/L BA and 15%v/v coconut water yielded 14 shoots in *Adhatoda vasica* (Bimal and Shahnawaz, 2012) whereas 15 shoots per node was observed in MS medium augmented with 1.5 mg/L BA for *Alternanthera sessilis* (Gnanaraj *et al*., 2011).BA was more effective in inducing a response and multiple shoot formation from nodal explants than Kn. Similar observations with respect to response was reported in *Justicia gendarussa* (Thomas and Yoichiro, 2010), *Beloperone plumbaginifolia* (Shameer *et al*, 2009) and response as well as multiple shoot formation in *Crossandra infundibuliformis* (Girija *et al*.,(1999) and *Mentha piperita* (Ghanti *et al*., 2004). The presence of NAA with BA reduced the percentage response and reduced the number of shoots than when BA was used alone, as also observed in *Mentha piperita* (Ghanti *et al*., 2004) and *Justicia gendarussa* (Thomas and Yoichiro, 2010). On the contrary, profuse growth of shoots from inflorescence tip explants of *Biophytum sensitivum* (Shivanna *et al*., 2009) was observed when MS media containing BA was enriched with 0.5 mg/L NAA.

In our study, with an increase or decrease in concentration of BA from over it's optimal level (3.0mg/L), shoot inducing ability and multiple shoot formation was decreased. The same was also reported in *Crossandra infundibuliformis* (Girija *et al*., (1999), *Andrographis paniculata* (Purkayastha *et al*., 2008), *Mentha piperita* (Ghanti *et al*., 2004) and *Phyllanthus amarus* (Shekhawat and Dixit, 2007). Full-strength and half-strength phytohormone-free MS media and media supplemented with varying concentrations of BA supported rhizogenesis in the present study, a phenomenon rarely observed among many of the *in vitro* regenerated plants. Nath and Buragohain (2005) reported the rooting of regenerated shoot tip explants *Adhatoda vasica* in MS basal media while Janarthanam and Sumathi (2010), in half strength MS media for *Justicia gendarussa*. Many of the regenerated plants such as *Astercantha longifolia* (Panigrahi *et al*., 2006), *Justicia prostrata* (Jeyachandran *et al*., 2010) produced roots in media supplemented with the auxin NAA, while those of *Stachytarpheta jamaicensis* (Rajender *et al*., 2012), *Adhatoda vasica* (Bimal and Shahnawaz, 2012) and *Andrographis lineate* (Deepa *et al.,*2011) rooted on media containing IBA. There are also studies that report *in vitro* derived shoots of *Mentha piperita* grown on full strength / half-strength medium containing BA and NAA rooted on hormone-free MS medium (Shasany *et al*., 2006), suggesting that rooting occurred in hormone-free medium when auxins were incorporated in shoot regeneration media. Thus, these observations suggest that there is a sufficient level of endogenous cytokinins and auxins

available in *Justicia wynaadensis* that support growth in phytohormone-free MS media, and BA at 3mg/L induces maximum multiple shoots from nodal explants in MS media. Regular sub culturing at intervals of 90 days is vital to prevent necrosis of the shoots which may be due to the large numbers of shoots regenerated, as well as due to diminishing nutrients with time. In conclusion, this paper describes a rapid method for multiple shoot regeneration from nodal explants of *Justicia wynaadensis* that is simple, economical and ensures a continuous supply of the plant, thus eliminating the need to harvest from the natural habitats that could arise due to commercial exploitation owing to its potential as a food supplement with cellular cholesterol lowering properties.

**Acknowledgement**

The authors are thankful to the Chairman of Presidency Group of Institutions, for providing part of the laboratory facilities to complete the work.

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