



# Improved in vitro regeneration, genetic fidelity analysis, antioxidant potential, and hairy root induction of *Justicia gendarussa* Burm. f

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## Abstract

*Justicia gendarussa* Burm. f. is an indispensable medicinal plant owing to its abundance of phytoconstituents and medicinal uses. In the study, we demonstrated the successful in vitro regeneration of *J. gendarussa* through nodal explants and its genetic and phytochemical analysis. A combination of 2 mg L<sup>-1</sup> BA + 0.1 mg L<sup>-1</sup> NAA + 15 mg L<sup>-1</sup> spermidine was found to be reliable in producing the maximum number of multiple shoots (16.6) and vigorous roots in Murashige and Skoog medium. Rooted shoots have been efficiently hardened and then slowly acclimatized to natural conditions. SPAR-based genetic fidelity checking revealed the true-to-type nature of in vitro regenerated plants with little variations by generating 105 bands through amplification of 15 primers. Furthermore, the protocol followed for in vitro culture was strengthened by the comparative determination of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Potential (AP). Methanolic extracts of in vitro plants showed higher amounts of TPC, TFC, and AP when compared to in vivo plants which might be due to the influence of plant growth hormones. Thus the in vitro regeneration protocol derived would vitally expedite large scale production of true to type plantlets of *J. gendarussa*. In addition, an attempt was made for the first time to devise a suitable protocol for the induction of hairy roots from this plant using two *Agrobacterium rhizogenes* strains which would lead to enhanced production of important anti-HIV metabolites of the plant such as gendarussin A, B and so on.

**Keywords** In vitro regeneration · *Justicia gendarussa* · Phytochemicals · SPAR markers · Spermidine

## Introduction

*Justicia gendarussa* Burm. f. commonly known as Black adusa is an evergreen shrub of the Acanthaceae family and found in a moist environment in tropical and sub-tropical parts of Asia. It is utilized to treat multifarious maladies such as chronic rheumatism, inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases, muscle pain, lumbago,

headache, earache, hemiplegia, leucoderma, asthma, nasal bleeding, bone fracture, and fever in traditional medicine (Chopra et al. 1986). Woradulayapinij et al. (2005) reported in vitro HIV type 1 reverse transcriptase inhibitor activity of this plant. Various studies witnessed the antiarthritic (Paval et al. 2009), anti-inflammatory, analgesic (Jothimaniyannan et al. 2010), larvicidal (Senthilkumar et al. 2009), antifungal (Sharma et al. 2011) and antibacterial (Sudhanandh et al. 2012) potentials of *J. gendarussa*. This plant is a rich source of potassium salts (Jayaweera 1980), minerals such as Ca, Mg & Zn (Corlett et al. 2002), phenolic dimmers called lignans which are the lead compounds for the development of antirheumatic agents (Mrunthunjaya and Hukkeri 2007) and triterpenoids. It has been found to contain alkaloids, triterpenoids, tannins, justicin, steroids, and flavonoids, gendarusin A and B. The major phytochemicals identified were lupeol,  $\beta$ -sitosterol, and  $\beta$ -sitosterol- $\beta$ -D-glycoside (Bachheti et al. 2011). The seeds of *J. gendarussa* show a very low germination percentage (Mrunthunjaya and Hukkeri 2007). Earlier, the plant was used to prepare traditional medicine

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by local people in small quantities, but commercialization of plant-based drugs in recent years has increased the demand and consequent exploitation of the plant. The uncontrolled collection and lack of organized cultivation have posed a severe threat to the natural germplasm of this important plant species. There is an urgent need to tackle the increasing demand for plant material besides conservation through biotechnology-based interventions.

Plant tissue culture serves as a savior of such plants whose natural stock is fast depleting and helps generate a large number of propagules for conservation, rehabilitation, commercial propagation, and for bioactive compound production (Georgiev et al. 2011; Wang et al. 2013). Moreover, the additional benefit for biomass production, the yield of secondary metabolites, has created a sensation to explore the regeneration potential of many medicinal plants in recent years (Dhir and Shekhawat, 2014; Sharma et al. 2014). However, still, the efficacy of tissue culture techniques depends upon the maintenance of the genetic integrity of in vitro raised plants since genetic stability cannot be guaranteed in the tissue culture-raised plants due to the epigenetic defects arising via somaclonal variation (Jin et al. 2008; Peyvandi et al. 2009). Hence examination of genetic stability of in vitro raised plants is of prime importance for commercial usage of the technique for large-scale production of high-quality clonal plants. SPAR (Single Primer Amplification Reaction) approach comprising of Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), and Directed Amplification of Minisatellite DNA (DAMD) molecular markers is very dynamic in detecting DNA based alterations since they are safe and cost-effective due to non-usage of radioactive probes (Mehrotra et al. 2012) and they require only a small quantity of DNA sample without any prior sequence information to design the primer.

Previously, preliminary studies were made in the in vitro propagation of *J. gendarussa*. Callus-mediated plant regeneration in *J. gendarussa* was reported by Agastian et al. (2006) and Thomas and Yoichiro (2010). Janarthanam and Sumathi (2010) reported shoot regeneration through nodal segments by supplementing coconut milk along with plant growth regulators and Kumar et al. (2012) made an attempt to regenerate shoots. As callus cultures are more prone to genetic alterations due to their undifferentiated status (Borchert et al. 2007), standardizing reproducible protocols for high-frequency direct regeneration is imperative for instigating biotechnology-based research studies in this anti-HIV medicinal plant. Moreover, no efforts are yet made to assess the genetic and chemical fidelity of in vitro plantlets.

The plant of our study has been identified with many medicinally valuable phytochemicals especially Gendarusin A, a flavonoid glycoside with anti-HIV activity on plasma blood of HIV patients in vitro (Hikmawanti et al. 2020). Devising protocols for increased metabolite production

would be the need of the hour for meeting the commercial large-scale demand of the plant extract. *Agrobacterium rhizogenes* mediated hairy root culturing offers a remarkable platform for enhanced production of metabolites in large quantities and serves as a model system for exploring metabolite synthesis and pathways. Therefore the objectives of the present study are (i) to achieve high frequency direct plant regeneration of *J. gendarussa* and to examine the genetic integrity of in vitro plantlets through the SPAR method as a plant conservatory method (ii) to compare the total phenolics, total flavonoids and antioxidant potential of in vitro regenerated plantlets with the mother plant and (iii) to induce hairy roots using *Agrobacterium rhizogenes* strains as an attempt to enhance the quantity of Gendarusin A and other metabolites of the plant.

## Materials and methods

### Plant material, surface sterilization and culture conditions

Vegetative stem cuttings of *J. gendarussa* were collected from Kerala, and mother plants were maintained in the shade house of the Department of Biotechnology, Alagappa University, Karaikudi. Auxiliary buds were initially treated with 1% bavistin for 20 min, repeatedly washed in running tap water and surface sterilized with 70% ethanol for 30 sec and 0.1% (w/v) mercuric chloride for 4 min. After thorough washing with sterile distilled water four times and blot drying with sterile tissue paper, the explants were inoculated on a Murashige and Skoog (MS) medium containing 3% sucrose (w/v) and 0.8% agar (w/v). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. All the cultures were placed in the culture room at 25±2 °C under a 16/8h photoperiod with a light intensity of 70 μ mol m<sup>-2</sup> s<sup>-1</sup> provided by white cool fluorescent lamps (Philips, India). A set of 12 explants per treatment was used and each experiment was repeated three times.

### Effect of auxin-cytokinin on in vitro regeneration

To determine the optimal plant growth regulators for efficient in vitro regeneration, the cultures were established in MS medium supplemented with different concentrations (0.5, 1, 1.5 and 2 mg L<sup>-1</sup>) of cytokinins 6-Benzyl amino purine (BA) and Kinetin (KN) combined with two auxins, Indole -3-acetic acid (IAA) and Naphthalene acetic acid (NAA) at 0.1 mg L<sup>-1</sup>. The MS medium without plant growth regulators was used as a control. Cultures were retained for four weeks, and growth parameters such as the frequency of explants causing shoot induction, the mean number of

shoots and the mean number of roots formed per explant were recorded.

### Effect of additives on multiple shoot induction

The *in vitro* shoots obtained from this intact nodal culture after four weeks of incubation were excised, and nodes from these were cultured further to induce multiple shoot buds. *in vitro* derived nodes were cultured in MS medium supplemented with different concentrations of various additives such as coconut water (5, 10, and 15%), adenine sulphate (10, 20, and 30 mg L<sup>-1</sup>), spermidine (10, 15 and 20 mg L<sup>-1</sup>) and phloroglucinol (100, 150 and 200 mg L<sup>-1</sup>) along with suitable plant growth regulators for the regeneration of multiple shoot buds. The MS medium without additives was used as a control. After four weeks of culture, the mean number of shoots, their length, and biomass were recorded.

### Hardening

Well-rooted *in vitro* plantlets were initially transferred to plastic cups filled with red sand and vermicompost (1:1) and placed in a plant growth chamber after washing the agar thoroughly in water. MS basal liquid medium was poured regularly once in three days for one month. After this, moderately grown plantlets were shifted to pots, placed in the plant growth chamber for two weeks, and then moved to the green house.

### Genetic fidelity analysis

Genomic DNA was extracted from mother plants and five randomly selected *in vitro* plantlets (after two sub cultures) using a HiPurA DNA isolation kit (Himedia, Mumbai, India). The quality and quantity of DNA were ascertained by agarose gel electrophoresis and spectrophotometer (Shimadzu, Japan). The clonal nature of the *in vitro* plants was examined by subjecting their DNA to the SPAR approach. Totally 15 primers, i.e. five primers for each of the three molecular markers (RAPD, ISSR, and DAMD) were used in this study, and they were synthesized by Sigma (St. Louis, USA). 25 µl of PCR mixture contained 50 ng of template DNA, 0.4 µM primer, 1X PCR buffer, 1 U Taq DNA polymerase, 200 µM of each of dNTPs, and Milli Q water. Amplification was carried out in a thermal cycler (Eppendorf, Deutschland, Germany) as follows: initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 37 °C for RAPD and 55 °C for ISSR and DAMD and extension at 72 °C for 2 min. The final extension was done for about 7 min at 72 °C. Amplification products were separated in a 1.5% agarose gel electrophoresis along with a 1 kb size marker. After staining with ethidium bromide, the banding profiles were documented using

a gel documentation system (Gel Doc XR, Bio-Rad, Quarry Bay, Hong Kong).

### Biochemical analysis

In order to study the role of plant growth regulators in Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Antioxidant Potential (AP) of *J. gendarussa*, *in vitro* plants regenerated in three different hormonal combinations namely IV1 (MS + 2 mg L<sup>-1</sup> KN + 0.1 mg L<sup>-1</sup> NAA), IV2 (MS + 2 mg L<sup>-1</sup> BA + 0.1 mg L<sup>-1</sup> NAA) and IV3 (MS + 2mg L<sup>-1</sup> BA + 0.1 mg L<sup>-1</sup> NAA 15 mg L<sup>-1</sup> + spermidine) and *in vivo* derived whole plants were shade dried and powdered. 10 g powder was extracted with 100 ml of methanol for three days at room temperature. The extract was filtered through Whatman filter paper and concentrated it in a rotary evaporator.

### Total phenolic content (TPC)

TPC in methanol extracts of *J. gendarussa* was determined by the Folin–Ciocalteu method (Lin et al. 1999). To the reagent mixture containing 0.5 ml Folin–Ciocalteu's phenol and 1.5 ml saturated sodium carbonate (20%), 100 µl of the extract was added. After mixing well, the tubes were kept in the dark at 25 ± 2 °C for 2 h for color development. Then absorbance was read at 765 nm using UV–Vis Spectrophotometer (Shimadzu, Japan). The standard curve was prepared by measuring the absorbance of the reagent mixture using different concentrations of gallic acid. The total phenolics in different samples in triplicates were determined from the regression curve and expressed in gallic acid equivalent per milligram of dry weight (µg GAE/mg DW).

### Total flavonoid content (TFC)

TFC assay was measured by the aluminum chloride assay by following the protocol of Marinova et al. (2005). The standard curve was prepared using quercetin as standard and absorbance was taken at 510 nm. Total flavonoid content was expressed as mg quercetin equivalent (µg QRE/mg DW) of extract. Samples were analyzed in triplicates.

### Antioxidant potential (AP)

DPPH radical scavenging activity of the methanolic extracts of the mother plant and *in vitro* plants was measured using the method of Blois (1958). One ml of 0.1 mM methanolic solution of DPPH (Sigma, St. Louis, USA) was added with 3 ml of methanolic extracts of *J. gendarussa* (100–500 µg mL<sup>-1</sup>), and the reaction mixture was allowed to react in the dark for 30 min. The absorbance was measured at 517 nm using UV–Vis Spectrophotometer (Shimadzu, Japan), and

Butyl hydroxytoluene ( $20\text{--}100\ \mu\text{g mL}^{-1}$ ) was used as standard. The antiradical activity was expressed as  $EC_{50}$  defined as the concentration of a sample ( $\mu\text{g mL}^{-1}$ ) at which 50 % of maximum scavenging activity was recorded. All the values were taken in triplicates.

### ***Agrobacterium rhizogenes* mediated transformation**

Leaves of 15-day-old tissue culture (TC) plants of *J. gendarussa* maintained on MS medium supplemented with  $2\ \text{mg L}^{-1}$  KN,  $0.1\ \text{mg L}^{-1}$  NAA, and  $15\ \text{mg L}^{-1}$  spermidine were used as explants for *A. rhizogenes* transformation. Two different explants (leaf blade and leaf with petiole) were wounded with a scalpel and pre-incubated in MS solid medium containing  $100\ \mu\text{M}$  acetosyringone for 1–2 days in dark prior to infection. 24 hours cultures of *A. rhizogenes* (10 ml) were centrifuged at 10000 rpm for 10 min, and the pellet formed was re suspended in MS basal liquid medium in order to obtain different culture ODs such as 0.5 and 1. About  $20\ \mu\text{l}$  of the diluted cultures were added over each explant and kept in the dark for 2–4 days for co-cultivation. Control explants were also incubated without adding bacterial cultures. After co-cultivation, all the explants were washed thoroughly with each  $500\ \text{mg L}^{-1}$  of ampicillin and cefotaxime for 30 min and rinsed four to five times with sterile distilled water. After blotting the explants in sterile tissue paper, they were inoculated on an MS basal medium containing each  $250\ \text{mg L}^{-1}$  of ampicillin and cefotaxime and incubated in the dark for hairy root induction. Two *Agrobacterium rhizogenes* strains namely A4 and MTCC 532, were used in this study.

### **Statistical analysis**

All experiments were performed in completely randomized design comprising 25 explants for each treatment, and each experiment was repeated thrice. Results were presented as mean  $\pm$  standard error of three independent experiments. Data were analyzed using one-way analysis of variance (ANOVA), and significant differences were determined by Duncan's Multiple Range Test (DMRT) at  $P \leq 0.05$ . All statistical analyses were conducted using the SPSS software (v.17.0) package.

## **Results and discussion**

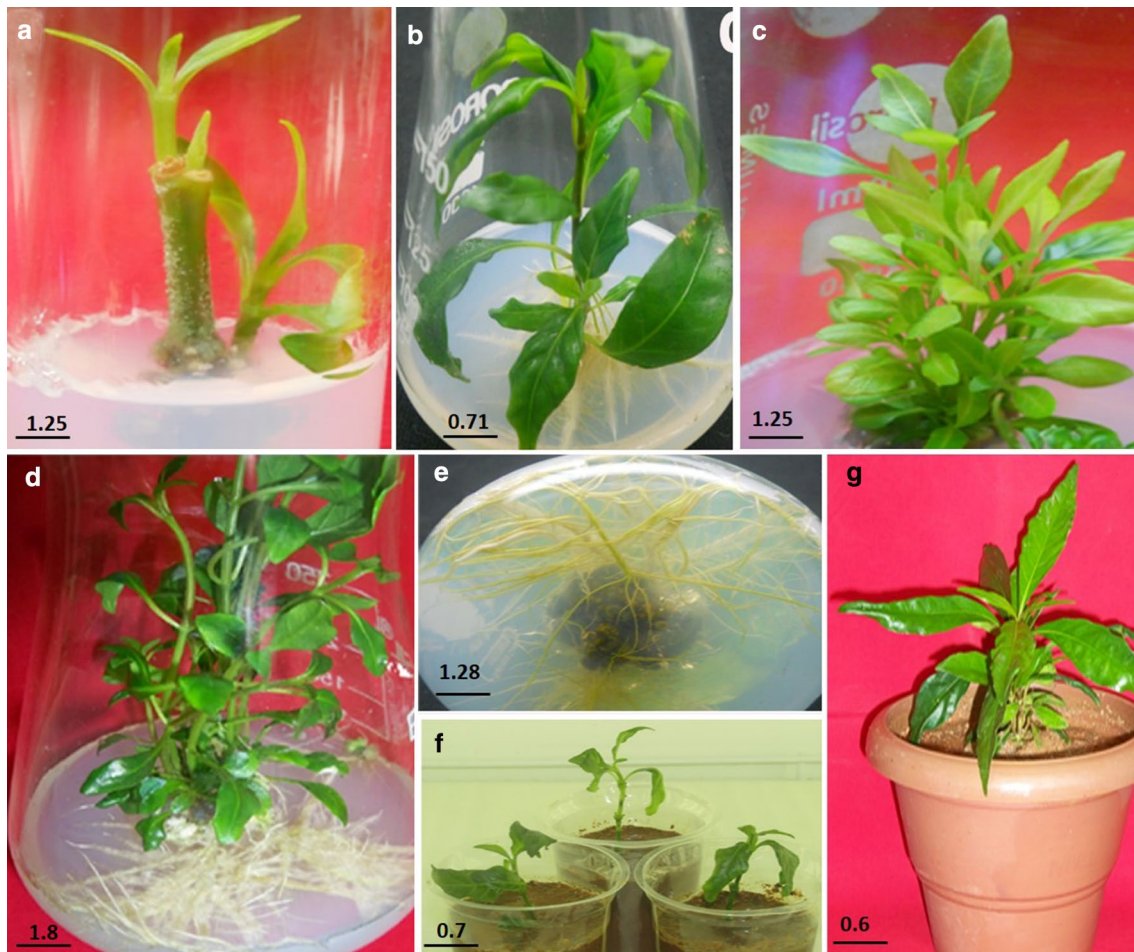
### **Synergistic effect of cytokinins and auxins on in vitro regeneration**

Nodal segments inoculated on MS medium without plant growth regulators (control) remained green but showed no sign of shoot induction. Instead, the nodal explants on MS

medium containing hormones sprouted within 8–10 days (Fig. 1a) and a marked influence was noticed in the shoot development depending on the nature and concentration of cytokinin. BA was superior to KN in shoot bud induction from nodal segments of *J. gendarussa*. Dhir and Shekawat (2014) pointed out that when compared to other cytokinins, BA has a stable structure and plant cells are able to metabolise it quickly and easily. It also stimulates the synthesis of endogenous cytokinin like zeatin which eventually promotes better shoot bud induction (Malik et al. 2005). The efficiency of BA in plant regeneration was well documented in several medicinal plants such as *Blumea mollis* (Tamilarasi and Thirugnanasampandan 2014), *Decalepis hamiltonii* (Sharma et al. 2014), *Salvadora oleoides* (Phulwaria et al. 2014) and so on.

Previously, Thomas and Yoichiro (2010) showed 87% of shoot induction frequency from nodes of *J. gendarussa* by culturing them on MS medium supplemented with  $4\ \text{mg L}^{-1}$  BA. However, in this study, we could able to obtain an increased (97.2%) frequency of shoot induction by using a lower concentration of cytokinin ( $2\ \text{mg L}^{-1}$  BA) in combination with  $0.1\ \text{mg L}^{-1}$  NAA. Further increase or decrease in the concentration of BA reduced the response percentage. Next to this, about 91.66% of explants revealed shoot initiation when cultured with  $2\ \text{mg L}^{-1}$  KN and  $0.1\ \text{mg L}^{-1}$  NAA. Among the auxins tested, NAA was found to be most effective compared to IAA. Both the frequency of shoot induction and the mean-number of shoots per explant were reduced on using  $0.1\ \text{mg L}^{-1}$  IAA compared to  $0.1\ \text{mg L}^{-1}$  NAA (Table 1). This is evidenced by the fact that among auxins, NAA is known to easily move across the cell membrane, resulting its rapid accumulation in plant cells (Nordstrom et al. 2004). The auxin/cytokinin ratio in the culture medium plays a crucial role in the morphogenetic response of cultured tissue. Various earlier reports suggest that NAA at lower concentrations along with the comparatively higher concentration of BAP have a critical role in plant regeneration, such as *Dendrocalamus hamiltonii* (Agnihotri et al. 2001), *Accacia catechu* (Kaur et al. 2004), *Clitoria ternatea* (Rout and Das 2004), and *Mucuna pruriens* (Faisal and Anis 2007), *Habenaria edgeworthii* (Giri et al. 2012).

In addition, this auxin-cytokinin combination provoked a better response by producing an increased mean number of shoots and roots. NAA at  $0.1\ \text{mg L}^{-1}$ , when supplemented along with BA, gave rise to 4.66 shoots (Fig. 1b). This hormonal combination has produced vigorous rooting with thick, long, and robust roots (Fig. 1c). However, the shoot multiplication rate in very minimal, and further addition of nutritive and growth-promoting substances is essential to induce multiple numbers of shoots from nodes of *J. gendarussa*.



**Fig. 1** Direct regeneration of *J. gendarussa* through nodal explants **a** Shoot bud induction from nodal explants after 10 days of inoculation **b** Shoot growth on MS medium supplemented with 2 mg L<sup>-1</sup> KN + 0.1 mg L<sup>-1</sup> NAA **c** Shoot and root growth on MS + 2 mg L<sup>-1</sup> BA + 0.1 mg L<sup>-1</sup> NAA after 28 days of culture **d** Induction of multiple shoots on using 15 mg/L spermidine along with MS + 2 mg L<sup>-1</sup> BA

+ 0.1 mg L<sup>-1</sup> NAA **e** Fully grown plantlet from multiple shoots after 28 days **f** Closer view of vigorous roots produced on using 15 mg/L spermidine along with MS + 2 mg L<sup>-1</sup> BA + 0.1 mg L<sup>-1</sup> NAA **g** Transfer of in vitro derived rooted shoots in plastic cups containing red sand and vermicompost **h** Hardened plantlet of *J. gendarussa* in a pot

### Effect of growth additives on multiple shoot induction

The number of shoots induced per explants, shoot length, and the biomass of in vitro grown whole plant varied with different additives tested. Amongst all additives, spermidine exerted a significant improvement in the shoot proliferation of *J. gendarussa*. The inclusion of spermidine (15 mg L<sup>-1</sup>) along with BA 2 mg L<sup>-1</sup> and NAA 0.1 mg L<sup>-1</sup> in medium proved to be critical with the highest number of multiple shoots (16 shoots/explant) with a shoot length of about 15 cm after 4 weeks (Table 2). Although MS medium containing BA and NAA (control) induced shoot regeneration (3.66 shoots/explant), the number of multiple shoots was enhanced by 4.5 times when combined with spermidine at 15 mg L<sup>-1</sup> (Fig. 1d). This particular additive helped to produce a 3.5

times increased shoot length of 15 cm compared with the control shoot length of 4.3 cm. Further increase or decrease in spermidine concentration decreased the production of multiple shoots. Next to this, phloroglucinol boosted multiple shoot proliferation but not to the extent of spermidine. Other additives such as coconut water and adenine sulphate did not influence the multiple shoot production in *J. gendarussa* (Table 2). Spermidine, a polyamine is a naturally occurring low molecular weight, an aliphatic nitrogenous compound involved in many critical cellular processes such as cell division, protein synthesis, DNA replication, and response to abiotic stress (Kakkar and Sawhney 2002). It interacts with phytohormones, acts as plant growth regulator or secondary hormonal messengers, and is a reserve of carbon and nitrogen in culturing tissues (Couee et al. 2004). It also brings out a drastic improvement in differentiation

**Table 1** Effect of cytokinins on shoot induction from nodal explants of *J. gendarussa* after 4 weeks of culture

Plant growth regulators (mg L <sup>-1</sup> )				Shoot induction frequency (%)	Mean no. of shoots	Mean length of shoots (cm)	Mean no. of roots
BA	KN	IAA	NAA				
–	–	–	–	–	–	–	–
1	–	0.1	–	62.66 ± 0.51 <sup>a</sup>	1.00 ± 0.00 <sup>c</sup>	2.30 ± 0.26 <sup>a</sup>	10.00 ± 0.89 <sup>a</sup>
2	–	0.1	–	72.33 ± 0.89 <sup>g</sup>	2.33 ± 0.51 <sup>e,f</sup>	3.83 ± 0.15 <sup>c</sup>	16.33 ± 1.36 <sup>e</sup>
3	–	0.1	–	86.66 ± 0.51 <sup>a</sup>	3.00 ± 0.00 <sup>a,b</sup>	3.40 ± 0.32 <sup>b</sup>	12.66 ± 1.86 <sup>b,c</sup>
–	1	0.1	–	42.11 ± 1.03 <sup>c</sup>	0.66 ± 0.51 <sup>b</sup>	2.06 ± 0.10 <sup>a</sup>	13.00 ± 1.78 <sup>c</sup>
–	2	0.1	–	69.33 ± 0.89 <sup>b</sup>	1.66 ± 0.89 <sup>d</sup>	3.13 ± 0.20 <sup>b</sup>	17.33 ± 2.25 <sup>f</sup>
–	3	0.1	–	53.33 ± 0.51 <sup>a</sup>	1.00 ± 0.00 <sup>c</sup>	2.16 ± 0.25 <sup>a</sup>	12.66 ± 1.86 <sup>b,c</sup>
1	–	–	0.1	81.33 ± 0.51 <sup>a</sup>	3.00 ± 0.00 <sup>a,b</sup>	3.16 ± 0.26 <sup>b</sup>	17.00 ± 2.25 <sup>f</sup>
2	–	–	0.1	97.33 ± 0.51 <sup>a</sup>	4.66 ± 0.89 <sup>e</sup>	4.33 ± 0.40 <sup>c</sup>	22.66 ± 2.25 <sup>g</sup>
3	–	–	0.1	89.33 ± 1.03 <sup>c</sup>	3.66 ± 0.51 <sup>c</sup>	3.66 ± 0.26 <sup>b</sup>	12.00 ± 1.54 <sup>b</sup>
–	1	–	0.1	70.66 ± 0.89 <sup>b</sup>	2.66 ± 0.51 <sup>f</sup>	2.50 ± 0.17 <sup>b</sup>	10.00 ± 0.89 <sup>a</sup>
–	2	–	0.1	92.00 ± 0.51 <sup>a</sup>	4.00 ± 0.51 <sup>f</sup>	3.23 ± 0.22 <sup>b</sup>	15.33 ± 1.36 <sup>d</sup>
–	3	–	0.1	78.66 ± 0.89 <sup>b</sup>	3.33 ± 1.03 <sup>b,c</sup>	2.93 ± 0.36 <sup>b</sup>	16.33 ± 1.36 <sup>e</sup>

Means within each column followed by the same letter are not significantly different at  $P < 0.05$  using DMRT

**Table 2** Influence of different additives along with MS medium + BA (2 mg L<sup>-1</sup>) + NAA (0.1 mg L<sup>-1</sup>) on multiple shoot proliferation from nodal explants of *J. gendarussa* after 4 weeks of culture

Additives	Concentration	Mean number of shoots	Mean length of shoots (cm)	Biomass of the plant (g)
Control	–	3.66 ± 0.51	04.33 ± 0.40 <sup>a</sup>	07.45 ± 0.38 <sup>a</sup>
Coconut water (%)	5	5.00 ± 0.89 <sup>d</sup>	04.10 ± 0.15 <sup>a</sup>	10.03 ± 0.78 <sup>a</sup>
	10	5.33 ± 0.51 <sup>d</sup>	06.10 ± 0.32 <sup>c</sup>	11.56 ± 0.71 <sup>a</sup>
	15	4.33 ± 0.51 <sup>b</sup>	04.76 ± 0.22 <sup>a</sup>	09.56 ± 0.36 <sup>a</sup>
Adenine sulphate (mg L <sup>-1</sup> )	10	2.66 ± 0.51 <sup>a</sup>	05.13 ± 0.20 <sup>b</sup>	06.90 ± 0.32 <sup>a</sup>
	20	4.66 ± 1.03 <sup>c</sup>	06.96 ± 0.40 <sup>d</sup>	08.10 ± 0.32 <sup>a</sup>
	30	2.66 ± 0.89 <sup>a</sup>	05.73 ± 0.22 <sup>b,c</sup>	07.75 ± 0.47 <sup>a</sup>
Spermidine (mg L <sup>-1</sup> )	10	7.66 ± 1.36 <sup>g</sup>	09.83 ± 0.54 <sup>f</sup>	17.60 ± 1.65 <sup>a</sup>
	15	16.66 ± 0.51 <sup>i</sup>	15.03 ± 0.59 <sup>h</sup>	26.33 ± 1.76 <sup>a</sup>
	20	11.33 ± 0.89 <sup>b</sup>	11.10 ± 0.26 <sup>g</sup>	20.40 ± 1.90 <sup>a</sup>
Phloroglucinol (mg L <sup>-1</sup> )	100	5.66 ± 1.03 <sup>e</sup>	07.86 ± 0.13 <sup>e</sup>	12.60 ± 1.72 <sup>a</sup>
	150	6.66 ± 0.51 <sup>f</sup>	08.03 ± 0.45 <sup>e</sup>	15.26 ± 0.59 <sup>a</sup>
	200	7.00 ± 0.89 <sup>b</sup>	06.76 ± 0.22 <sup>c</sup>	13.20 ± 1.56 <sup>a</sup>

and plant regeneration (Scholten 1998). Spermidine was reported to induce multiple shoot production and regeneration in many medicinal and crop plants. Recently Arun et al. (2014) reported the maximum percentage of response (96.94 %) with 39.02 shoots/explant in soybean by adding 137.69 μM of spermidine. In 2011, Sivanandhan et al. produced 46 shoots/explant in *Withania somnifera* by supplementing 20 mg L<sup>-1</sup> of spermidine along with desired plant hormones. In *J. gendarussa*, we have also witnessed a marginal increase (3.5 times) in biomass of the plant grown in spermidine-containing medium (26.3 g) when compared to the control plant (7.45 g). After four weeks of the culture period,

well-grown plantlets of *J. gendarussa* with vigorous shoots and roots were developed. The addition of this polyamine has also stimulated vigorous root production (Fig. 1e), and this has averted the need for a separate rooting process in this particular plant.

Hence a simple and one-step protocol has been derived for in vitro propagation of *J. gendarussa* using axillary buds in MS medium augmented with 2 mg L<sup>-1</sup> BAP, 0.1 mg L<sup>-1</sup> NAA and 15 mg L<sup>-1</sup> spermidine within the culture period of four weeks. Well-grown in vitro plants after three sub cultures were systematically subjected to acclimatization. Initially, they were maintained in a growth chamber

in plastic cups (Fig. 1g), and after two to three weeks, they were shifted to pots (Fig. 1h) and placed in the green house. Hardened plants exhibited an 80% survival rate (data not shown) with similar morphology as the mother plant's.

### Genetic integrity analysis

As in vitro plantlets are prone to culture-induced genetic variability, which can be inherited to next generations, it is crucial to launch the stable genetic nature of micro propagated plants for its efficient commercial utility (Bhatia et al. 2011). These somaclonal variations may be derived from changes, chromosomal rearrangements, polyploidy, and gene mutations (Palombi and Damiano 2002). In this study, the stable genetic nature of regrown plantlets was assessed by utilizing the SPAR method comprising of RAPD, ISSR, and DAMD markers. Totally 15 primers (5 from each type) were involved in the amplification of genomic DNA of five randomly selected one month-old in vitro grown *J. gendarussa* plants and one mother plant. Table 3 summarizes the details of primers used, their sequences, and the number of bands produced. 5 RAPD primers produced a total of 29 bands, among which primer OPH 03 has generated a maximum of 10 bands within the size range of 300–1200 bp. In the case of ISSR, the number of bands obtained by each primer varied from 4 (HB9) to 6 (HB 13 and A15), and a sum of 26 bands was produced by 5 ISSR primers. Out of the three markers, DAMD primers produced a maximum of 50 bands within the size range of 300 to 1500 bp. On the whole, 105 efficient, clear, well resolved and reproducible bands were created from 15 primers. The number of bands scored by each

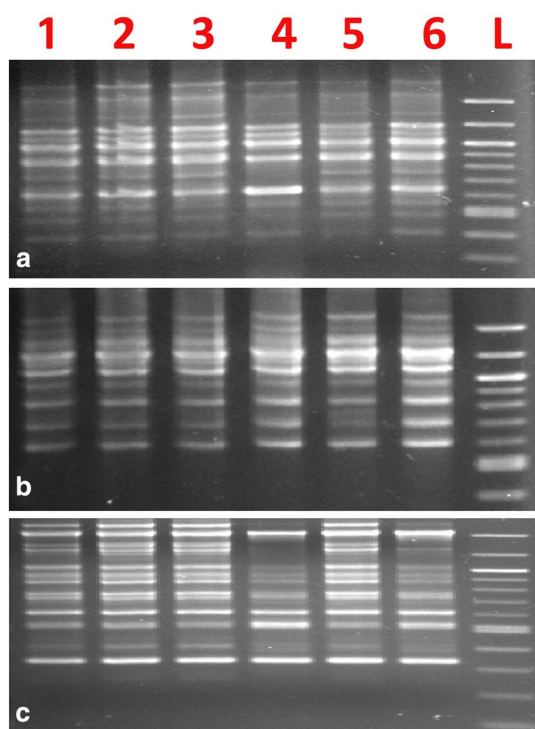
primer ranged from 4 (OPH 02, OPG 03, and HB 9) to 13 (6.2H) with an average of 10.5 bands per primer (Table 3). The banding profiles generated by 15 primers of all five regrown plants were monomorphic to the mother plant, and only plant 4 showed little genetic variation on using the DAMD primer 6.2 H. Overall, the genetic similarity was 98 % and the gel pictures showing the banding patterns of three primers were displayed in Fig. 2. Thus there is very little polymorphism that too observed in one plant and can be efficiently screened and excluded from large scale commercial production. A similar type of study using the SPAR technique for analyzing genetic stability of micro propagated plantlets were carried out in *Kaempferia galanga* (Mohanty et al. 2011) and in *Solanum trilobatum* (Shilpha et al. 2014).

### Comparative determination of total phenolics, flavonoids, and antioxidant potential

It is imperative to check the chemical fidelity of the in vitro regenerants as the soma clonal variations tend to alter the secondary metabolites production of plants. As phenolics and flavonoids represent basic phyto-chemicals of a plant, we tend to estimate its quantity in the in vitro regenerated plants and compared with the wild plants. Our results vividly represent no negative effect on secondary metabolites production. Results obtained clearly showed a strong correlation between TPC, TFC, AP, and plant hormones. Both TPC and TFC were higher in in vitro derived plant extracts ( $47.61 \pm 1.32 \mu\text{g GAE/mg DW}$ ;  $256.62 \pm 0.74 \mu\text{g QRE/mg DW}$ ) when compared to the mother plant extract ( $21.79 \pm 1.85 \mu\text{g GAE/mg DW}$ ;  $120.62 \pm 1.34 \mu\text{g QRE/mg}$

**Table 3** SPAR based genetic fidelity analysis of in vitro regenerants of *J. gendarussa* using 15 primers

S.No	Primer name	Primer sequence	Total number of bands	Size range (bp)
1.	RAPD OPH 02	TCGGACGTGA	4	400-750
2.	OPH 03	AGACGTCCAC	10	300-1200
3.	OPF 01	ACGGATCCTG	5	400-900
4.	OPF 04	GGTGATCAGG	6	300-800
5.	OPG 03	GAGCCCTCCA	4	800-1400
6.	ISSR HB9	(GT) <sub>6</sub> GG	4	500-1200
7.	HB13	(GAG) <sub>3</sub> C	9	400-1000
8.	A3	A(CA) <sub>7</sub> CC	5	300-1000
9.	A13	(CCG) <sub>6</sub>	5	300-800
10.	A15	(AC) <sub>9</sub> T	6	400-1200
11.	DAMD 6.2H	AGGAGGAGGGGAAGG	13	300-1500
12.	URP25F	GGACAAGAAGAGGATGTGGA	8	600-1500
13.	URP 30F	GGACAAGAAGAGGATGTGGA	12	400-1400
14.	URP 38F	AAGAGGCATTCTACCACCAC	9	700-1500
15.	FVIIex8	ATGCACACACACAGG	8	400-1000



**Fig. 2** Genetic similarity of in vitro plants of *J. gendarussa* as revealed by **a** RAPD - OPH03 **b** ISSR - HB13 **c** DAMD - 6.2H markers. Lane 1 mother plant, Lanes 2-6 in vitro regenerated plants, Lane L 100 kb ladder

DW). Among the three in vitro derived plant extracts, IV3 topped the list, thus revealing the role of BA and spermidine in increasing the secondary metabolites production (Table 4). A similar tendency was observed in the DPPH assay where IV3 extract exhibited a lower EC 50 value ( $55.36 \pm 0.55 \mu\text{g mL}^{-1}$ ) which means higher free radical scavenging capacity than those obtained with other in vitro and mother plant extracts.

This is evidenced by the fact that the exogenous supply of different plant growth regulators during the in vitro propagation stage considerably influenced the production of phenolic and flavonoids in in vitro-derived plants. It results in different antioxidant properties between in vitro

and conventionally propagated plants (Piateczak et al. 2014). On the contrary, higher TPC and AP in in vitro plants than in vitro plants have been documented in *Passiflora alata* by Lugato et al. (2014). However, similar to our results, a positive correlation between the phenolic content and the antioxidant activity was observed in *P. alata*, and this has corroborated the idea that the amount of phenolic compounds is a key factor for the antiradical activity. Arumugam et al. (2012) reported higher phenolic contents in the micro propagated plant leaves of *Excoecaria agallocha*. Increased level of secondary metabolites might be because the plants have undergone stress during in vitro culturing, which activates the genes involving the secondary metabolites production pathway.

### Hairy root induction through *Agrobacterium rhizogenes* mediated transformation

The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains, and signal molecules. Therefore, the following conditions for transformation have been optimized.

#### Culture OD

Bacterial concentration (at 600 nm) plays an important role in the production of transformed roots. Suboptimal concentrations resulted in low availability of bacteria for transforming the plant cells, while high concentrations decreased their potential by competitive inhibition in soybean *A. rhizogenes*-mediated transformation (Kumar et al. 1991). Optimum bacterial concentration was 1.0 in *Torenia fournieri*, which is similar to the effect of bacterial concentration on hairy root formation in soybean transformation, therefore possibly sharing the same mechanism (Tao and Li 2005). In some plants, the transformation was effective at low bacterial ODs. The culture OD was between 0.4-0.6 in the hairy root induction in tea leaves using MTCC 532 strain (John et al. 2009). Tao and Li (2005) had also studied the effect of different culture ODs on hairy root induction in *Torenia*

**Table 4** Comparative evaluation of total phenolics, flavonoids and antioxidant potential of methanolic extracts of mother plant and in vitro plants grown in different hormonal combinations

S.No.	Assay	Methanolic extract of wild plants	Methanolic extract of in vitro plants		
			IV1	IV2	IV3
1.	Total phenolics ( $\mu\text{g GAE/mg DW}$ )	$21.79 \pm 1.85^a$	$26.76 \pm 0.74^b$	$34.58 \pm 0.74^c$	$47.61 \pm 1.32^d$
2.	Total flavonoids ( $\mu\text{g QRE/mg DW}$ )	$120.62 \pm 1.34^a$	$133.68 \pm 0.58^b$	$172.83 \pm 1.57^c$	$256.62 \pm 0.74^d$
3.	Antioxidant potential EC <sub>50</sub> value ( $\mu\text{g mL}^{-1}$ )	$62.69 \pm 0.75^d$	$61.29 \pm 0.54^c$	$60.30 \pm 0.72^b$	$55.36 \pm 0.55^a$

Means within each column followed by the same letter are not significantly different at  $P < 0.05$  using DMRT



*fournieri*. Taking these into considerations, different culture ODs has been tested in this study for effective hairy root induction using MTCC 532 and A4 strains of *A. rhizogenes*. The explants have got severely damaged by using bacterial OD 1. On comparing ODs 0.5 and 1.0, hairy root induction is the highest in 0.5 culture OD in both the strains of *A. rhizogenes* (Fig. 3). Therefore 0.5 OD has been considered adequate for hairy root induction in *J. gendarussa*.

### Co cultivation duration

Co-cultivation is very important in the transformation process. Bacterial attachment, T-DNA transfer, and integration occur during this stage (Su et al. 2002). We can accelerate these processes by supplementing some ingredients in the co-cultivation medium or prolonging co-cultivation time so that these processes can terminate sufficiently. Our results indicated that if the co-cultivation time was too short, these transformation processes could not be completed. In contrast, long co-cultivation time could negatively affect transformation by reducing bacterial affinity to the plant cell or by competitive inhibition. More extended co-cultivation periods do not help in increase the transformation efficiency as it results in the overgrowth of the *Agrobacterium* strains on the explants, which then becomes difficult to control. In *Torenia fournieri*, the hairy root frequency increased progressively with increasing co-cultivation time from 2 to 4 days, becoming asymptotic towards the end. At more extended co cultivation periods, the problem of increased cell densities of bacteria in the selection medium had been faced. Therefore, co-cultivation was restricted to 2 or 3 days which was found to be sufficient for transformation (Tao and Li 2005). Two days was also confirmed as the optimum co-cultivation duration, whereas 3 or 4-day co-cultivation may cause the overgrowth of *A. rhizogenes* leading to damage of plant cells and consequently resulting in a low transformation frequency. 2 days co-cultivation also did not possess any

threat to explants (Fig. 4b). On the other hand, a short co-cultivation time may disrupt *A. rhizogenes* cell proliferation, thereby reducing its virulence and leading to a low transformation frequency. 2 days co-cultivation was found to induce the maximum number of hairy roots in *J. gendarussa* when compared with 1- or 3-days co-cultivation period (Fig. 3). Overgrowth of *A. rhizogenes* leading to damage of plant cells was observed in increased co-cultivation time of 4 or 5 days (Fig. 4c).

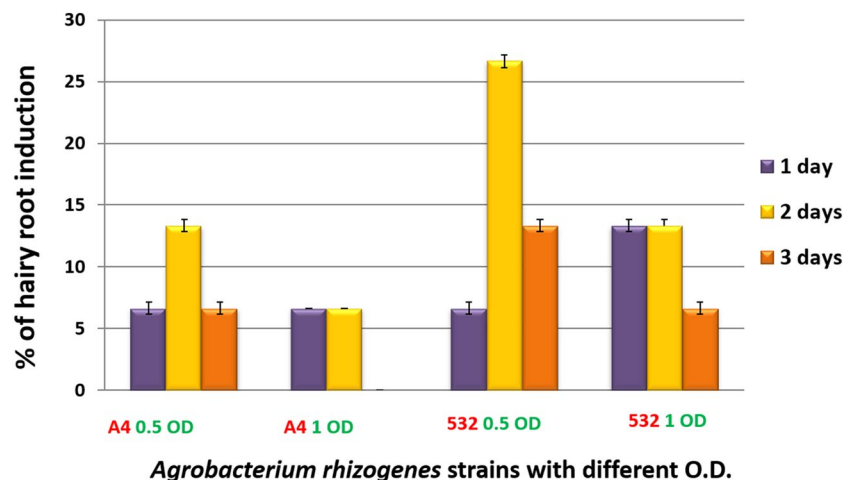
### Explant choice

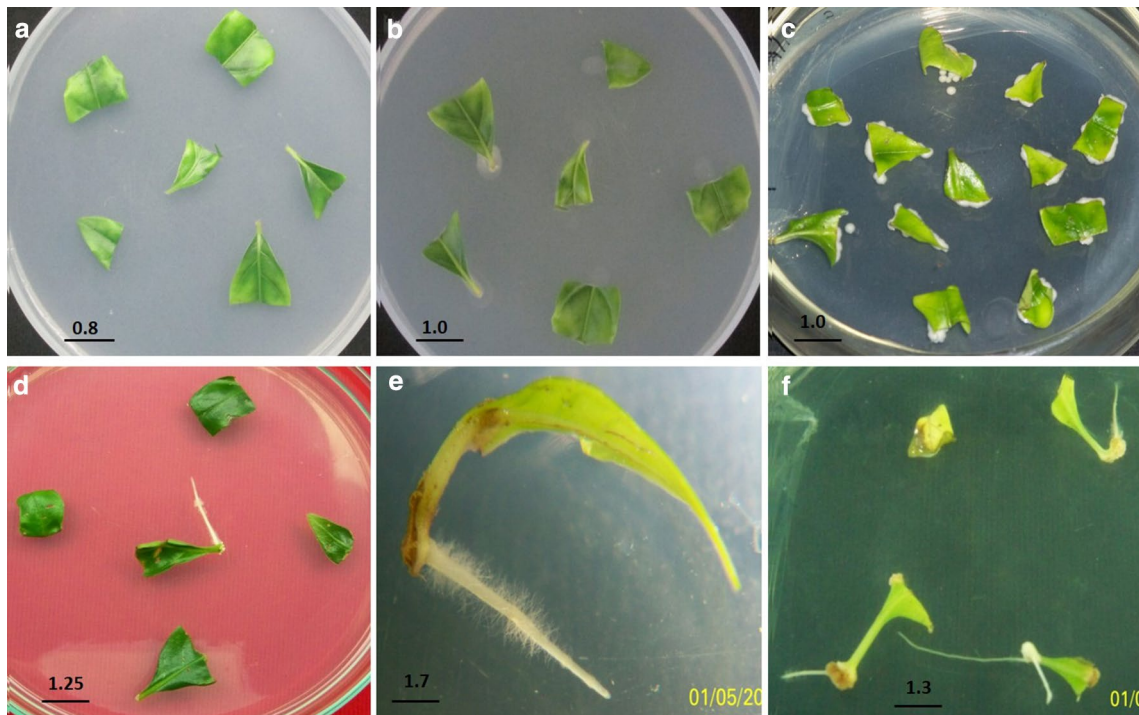
Leaf petiole explants proved to be relatively better than the leaf blade explants in terms of transformation frequencies and further growth potentials of the resulting hairy root clones. The frequency of hairy root induction and regeneration of transformed plants was higher in leaf petiole explants when compared to leaf blade explants. Hence for this medicinal plant, the leaf along with the petiole part is found to be very much efficient for hairy root induction (Fig. 4d, e, f). The imperative role of explants in determining the plant genotype-bacteria strain compatibility has received substantial research attention over the years.

### Hairy root induction using MTCC 532 and A4 strains

The methodology devised has successfully induced hairy roots from leaf petiole explants of *J. gendarussa*. Prior to transformation, the leaf petiole explants were separated from 20 days old in vitro plants and precultured in MS basal with 100  $\mu$ M acetosyringone for 2 days in the dark. Acetosyringone or related compounds functioning as signal molecules have been reported to improve the *Agrobacterium*-mediated transformation in several plant species (Hu and Alfermann 1993). Acetosyringone has been known to enhance transformation efficiency due to the activation of *vir* genes in *A. tumefaciens* (Gelvin 2000). Therefore, we presume that the

**Fig. 3** Hairy root induction from leaf petiole explants of *J. gendarussa* through *A. rhizogenes* mediated transformation **a** preculturing of explants on MS basal + 100  $\mu$ M acetosyringone for 2 days in dark, **b** Co cultivation of leaf petiole explants with 0.5 O.D. of *A. rhizogenes* strain, **c** overgrowth of *Agrobacterium* noticed during selection, **d** hairy root induced from petiole by MTCC 532 strain infection, **e** closer view of the hairy root induced by MTCC 532 strain, **f** Hairy roots induced by A4 strain





**Fig. 4** Effect of culture O.D. and co cultivation duration on hairy root induction in *J. gendarussa* on using A4 and MTCC 532 strains of *A. rhizogenes*

enhancement in transformation by acetosyringone treatment may be due to activation of *vir* genes which is absolutely required for the T-DNA delivery to plant tissues. Hairy roots induced from leaf petioles have been separated from the explants and allowed to grow independently in a fresh MS basal medium with suitable antibiotics. After achieving mass proliferation of hairy roots, they should be sub-cultured in such a way to withdraw the antibiotics in the growing medium by gradually reducing the concentration of the antibiotic in each sub culture. This will be followed by the molecular and chemical analysis of hairy roots for the presence of *rol* genes and the quantification of major phytochemicals respectively.

## Conclusions

Our investigation highlighted the high-frequency regeneration of the scented shrub *J. gendarussa* mediated by spermidine supplementation. Banding profiles obtained by the SPAR approach revealed 98% genetic similarity and greatly helped in eliminating plants with little genetic variations in the large-scale commercial cultivation. In addition, in vitro plant extracts recorded higher total phenolics, flavonoids, and anti-oxidant potential compared to the mother plant extract, which further strengthened the robust chemical nature of in vitro regenerants. The protocol provided the

basis for further investigation on germplasm conservation, cell suspension, transformation and metabolic engineering in this valuable medicinal plant. To our knowledge, this is the first report of its kind in describing direct regeneration followed by genetic and chemical fidelity assessment of *J. gendarussa*.

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