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PLANT TISSUE CULTURE

Genetic homogeneity assessment of *in vitro*-regenerated plantlets of *Nyctanthes arbor-tristis* L. and comparative evaluation of bioactive metabolites and antioxidant activity

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Abstract

In vitro propagation of *Nyactanthes arbor-tristis* L. was achieved by culturing *N*-phenyl-*N*′-benzothiazol-6-yl-urea (PBU)-pretreated nodal explants in Murashige and Skoog (MS) medium without any phytohormones. Pretreatment of nodal explants in liquid MS medium with 100 μM *N*-phenyl-*N*′-benzothiazol-6-yl-urea for 4 d showed the highest shoot proliferation by producing maximum number of shoots (17.40 ± 1.02) per explant, with average shoot length of 5.96 ± 0.08 cm at the end of 8 wk. Effective rooting was accomplished by preincubating the cut-end of shoots with half-strength MS medium containing 6 μM indole-3-butyric acid for 1 wk, followed by implantation into half-strength MS medium; an average of 6.20 ± 0.049 roots per shoot were produced. Seventy-eight percent of the plantlets regenerated *in vitro* were successfully acclimatized and transferred to soil. These plantlets appeared to be morphologically similar to the donor plants. The genetic fidelity of these *in vitro*-regenerated plantlets was confirmed by start codon targeted polymorphism (SCoT) marker analysis, followed by comparative evaluations of the bioactive metabolites (ursolic acid, rengyolone, arbortristoside-A, and nyctanthoside), antioxidant-rich phytochemicals, and radical scavenging activities. This optimized *in vitro* propagation protocol should be an aid for the conservation of *N. arbor-tristis* germplasm, as well as cater to the needs of herbal industries for the production of therapeutic molecules.

Keywords Antioxidant activity · Bioactive metabolites (arbortristoside-A, nyctanthoside, rengyolone, and ursolic acid) · *N*-Phenyl-*N*′-benzothiazol-6-yl-urea (PBU) · Start codon targeted polymorphism (SCoT) marker analysis

Introduction

Nyctanthes arbor-tristis L. (family Oleaceae), commonly known as "night flowering jasmine," is an important medicinal species. Although this plant is native to the Indian subcontinent, where it is distributed in the wild in sub-Himalayan regions, night flowering jasmine is also grown as an ornamental in subtropical gardens to cater to ritual and esthetic needs. This plant is a promising source of many therapeutic biomolecules, such as the iridoid glycosides arbortristosides, nyctanthoside, polyphenols, flavonoids, β-sitosterol, astragalin, nyctanthic acid, rengyolone, α-crocetin, and ursolic acid, among others (Tuntiwachwuttikul *et al.* 2003; Agrawal and Pal 2013; Khanapur *et al.* 2014; Saini *et al.* 2014). Plant-based extracts, derived either from leaves, flowers, seeds, roots, or bark of *N. arbor-tristis*, have been used in conventional medicine for the treatment of asthma, diuresis, cancer, rheumatism, sciatica, gout, malaria, filaria, liver dysfunction, skin diseases, and worm infection of intestine, because of their well-documented anti-arthritic, anti-malarial, anti-filarial, hepato-protective, anti-inflammatory, immuno-modulatory, anti-leishmanial, and antioxidant properties (Tuntiwachwuttikul *et al.* 2003; Rathee *et al.* 2007; Rani *et al.* 2012; Agarwal *et al.* 2013; Agrawal and Pal 2013; Saini *et al.* 2014; Mishra *et al.* 2016). Furthermore, some notable herbal preparations using plant parts of *N. arbor-tristis* are being sold today.

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To date, the demand for *N. arbor-tristis* plant parts for pharmaceutical, esthetic, and ritual activities is met by the natural plant population, but such unrestricted utilization of the species has resulted in the unchecked exploitation of a vulnerable resource and threatens to bring this species in the near future to the brink of extinction in its native habitat. This situation is further aggravated because seed-based propagation of night flowering jasmine is constrained by low seed viability, poor seed germinating ability, and delayed root system growth at the sapling stage (Rout *et al.* 2008; Jahan *et al.* 2011). Thus, there is a growing need to optimize *in vitro* propagation protocols for rapid multiplication and conservation of this multipotent medicinal plant (Phillips 2004).

During the last couple of decades, several attempts to optimize the few existing protocols for *in vitro* organogenesis of *N. arbor-tristis* have been made (Rout *et al.* 2008; Jahan *et al.* 2011; Sahu *et al.* 2012). Additionally, several non-purine phenyl urea derivatives with cytokinin activity, such as thidiazuron (TDZ), *N*-phenyl-*N*′-benzothiazol-6-yl-urea (PBU), and *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU), have been used to promote either adventitious organogenesis or somatic embryogenesis in different species (Ricci and Bertoletti 2008; Rolli *et al.* 2011; Li *et al.* 2015). Jahan *et al.* (2011) reported that preconditioning axillary buds of *N. arbor-tristis* with TDZ for 8 d and subsequent implantation in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) enhanced the rate of shoot multiplication *in vitro*, and similar reports have also been made for several related species. Alternatively, synthetic derivatives, such as PBU, have been tested in many species for *in vitro* propagation either *via* organogenesis or somatic embryogenesis (Torelli *et al.* 2006; Huang *et al.* 2010; Rolli *et al.* 2011; Carra *et al.* 2012; Li *et al.* 2015).

Considering the reported responses of plant cultures to PBU during organogenesis and somatic embryogenesis, in the present study, its application in the tissue culture of night flowering jasmine was investigated for an alternative propagation method, with the eventual goals of conservation of native *N. arbor-tristis* populations, while permitting production of bioactive metabolites on a commercial scale from propagated plants.

Furthermore, because genetic variation can arise during *in vitro* multiplication *via* organogenesis, whether as an expression of epigenetic imprints or due to the modifications in the genetic makeup induced by culture environments (Larkin and Scowcroft 1981), the genetic homogeneity of any resulting plantlets needed to be evaluated. The clonal fidelity of regenerated plantlets of *N. arbor-tristis* has not been assessed in any of the earlier studies, neither at the genetic level, nor in terms of the plant's bioactive, therapeutic molecules and phytochemical content.

In other plant species, various DNA markers have been used, either individually or in conjunction with profiling of bioactive molecules, for the assessment of genetic homogeneity in plantlets regenerated *in vitro* (Ghaderi and Jafari 2014; Adeniran *et al.* 2018). Among these, start codon targeted polymorphism (SCoT; Collard and Mackill 2009) markers have been used successfully in recent days for the evaluation of genetic homogeneity of *in vitro*-derived plantlets in *Dendrobium nobile* Lindl. (Bhattacharyya *et al.* 2014), *Alhagi maurorum* Medik. (Agarwal *et al.* 2015), *Helicteres isora* L. (Muthukumar *et al.* 2016), and *Abutilon indicum* (L.) Sweet (Seth *et al.* 2017). These SCoT markers are preferred, because the primers are designed for short conserved regions around the ATG start codon and its targeting sequences, and are therefore most likely part of a gene (Collard and Mackill 2009; Bhattacharyya *et al.* 2014). Obviously, from a pharmacological viewpoint, consistency in terms of quality and content of bioactive metabolites of *in vitro*-regenerated medicinal plants is crucial (Dörnenburg and Knorr 1995).

The present report describes an alternative protocol for the *in vitro* propagation *N. arbor-tristis* from pretreated nodal explants and provides an assessment of the genetic fidelity of the *in vitro*-regenerated plantlets using SCoT marker profiling, along with a comparative evaluation of their bioactive molecules, antioxidants, and radical scavenging activities.

Materials and Methods

Explant preparation, media, chemicals, and culture conditions Young and fresh apical stems of approximately1.5 cm length were obtained from 12-mo-old *N. arbor-tristis* (accession NAET-08) and were washed with water for 5 to 10 min, followed by 1% (*v/v*) Extran liquid detergent and 1% Bavistin® fungicide for 10 min each. Subsequently, truncated nodal explants were surface sterilized with 0.01% (*w/v*) $HgCl_2$ for 3 to 4 min, followed by three rinses with sterile double-distilled water under a laminar flow hood. Basal MS (Murashige and Skoog 1962) medium, containing various combinations of 0.0, 2.5, 5.0, 7.5, 10.0, or 15.0 μM α-naphthaleneacetic acid (NAA), 6-benzyladenine (BA), or kinetin (KIN), and growth additives (0, 7.5, 15.0, 22.5, or 30.0 μM glutamine, 0.0, 5.0, 10.0, 15.0, or 20.0 μM proline, and 0.0, 15.0, 30.0, 45.0, or 60.0 μM adenine sulfate), or 25.0, 50.0, 75.0, 100.0, or 125.0 μM PBU were further supplemented with 3% (*w/v*) sucrose; the pH was adjusted to 5.8 ± 0.1 using 1 N HCl before 0.8% (*w/v*) agar-agar was added, and the medium was autoclaved at 1.06 kg cm^{-2} for 15 to 30 min. All the cultures were maintained in culture room at 25 ± 2°C, 60–70% relative humidity and a 16-h photoperiod, under approximate photosynthetic photon flux of 40 μmol m^{-2} s^{-1} using fluorescent tubes (Crompton Greaves, Mumbai, India).

Shoot bud induction and proliferation of shoots *in vitro* The sterilized nodal explants were incubated on MS medium containing various combinations of 2.5, 5.0, 7.5, 10.0, or 15.0 μM BA, 2.5, 5.0, 7.5, 10.0, or 15.0 μM KIN, and 2.5, 5.0, 7.5, or 10.0 μM NAA, and the best responding plant growth regulator (PGR) combination, in terms of number of shoots per node *vis-à-vis* shoot length, was identified. This best combination of PGRs was further enriched with different concentrations and combinations of 15.0, 30.0, 45.0, or 60.0 μM adenine sulfate, 5.0, 10.0, 15.0, or 20.0 μM proline, and 7.5, 15.0, 22.5, or 30.0 μM glutamine, and their influence on shoot numbers per nodal explant was assessed.

In another set of experiments the nodal explants were pretreated with liquid MS medium containing either optimized concentrations of BA, KIN, NAA, and growth additives, or 25, 50, 75, 100, or 125 μM PBU for 2, 4, 6, and 8 d on a rotary shaker (Rivotek-50082001, Riviera Glass Private Limited, Mumbai, India) at 75 rpm, before being embedded in PGR-free solidified MS medium. The effects of different combinations of PGRs, growth additives, and pretreatments on regeneration response, number of shoots, and length of shoots after 8 wk of culture were recorded.

Rooting *in vitro* and acclimatization *In vitro*-derived shoots of about 4 to 5 cm in length were excised from the clumps of shoots, pretreated on half-strength MS medium supplemented with 2.0, 4.0, 6.0, 8.0, or 10.0 μM NAA or indole-3-butyric acid (IBA), individually, for 1 wk, before transfer to PGR-free half-strength MS medium. Data on rooting response, number of roots, and average root length were recorded after 3 wk. Well-rooted plantlets were washed with sterile double-distilled water and transferred to plastic pots (3 in. diameter, 350 ml total volume; OEM Ltd., Bhubaneswar, India) containing 1:1:1 (*v/v/v*) garden soil, soil-rite mix (Keltech Energies Ltd., Bengaluru, India), and vermi-compost. These potted plantlets were retained in the culture room at 25 ± 2°C for 1 wk and successively transferred to a net house and the experimental field, following the routine protocol of acclimatization (Sahu *et al.* 2012). All the plant growth regulators, growth additives and chemicals used for the media preparation were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Genetic fidelity analysis using SCoT markers Along with their *ex vitro* donor plant, 39 *in vitro*-regenerated plantlets were randomly selected from the best-responding culture combinations for the assessment of genetic uniformity. Fresh and young leaves (approximately 1.5 g) were ground in liquid nitrogen and genomic DNA was extracted using the modified cetyl trimethyl ammonium bromide (CTAB) method described by Mishra *et al.* (2013). The crude DNA was purified using RNAse and proteinase K (B. Genei, Bangalore, India) treatment, followed by successive washes of 25:24:1 (*v/v/v*) phenol:chloroform:isoamyl alcohol (Himedia Laboratories Pvt. Ltd., Mumbai, India), and 24:1 (*v/v*) chloroform:isoamyl alcohol (Himedia Laboratories Pvt. Ltd., Mumbai, India), as described previously by Seth *et al.* (2017). The thus purified DNA was precipitated by addition of chilled ethanol, pelleted, and dried under vacuum. Subsequently, the DNA sample of each plant was dissolved in 10 mM tris (hydroxymethyl) aminomethane (Tris): 1 mM ethylene-diamine tetraacetic acid (EDTA) buffer and equilibrated to a concentration of 20 ng μL^{-1}.

The polymerase chain reaction (PCR) mix of 25 μL total volume and containing 40 ng DNA, 2 mM $MgCl_2$, 10 mM dNTP mix, 2.5 μL of 10× assay buffer (100 mM Tris-Cl, pH 8.3; 0.5 M KCl; 0.1% (*w/v*) gelatin), 1 U *Taq* DNA polymerase (B. Genei, Bangalore, India), and 20 ng of SCoT primer(s) was amplified in a MyCycler thermal cycler (BioRad, Hercules, CA) programmed for 35 cycles, as described in Seth *et al.* (2017). The amplified products were electrophoretically separated on 1.4% (*w/v*) agarose gel using TAE (40 mM Tris acetate; 2 mM EDTA) buffer at a constant 50 V, visualized with 0.5 μg mL^{-1} ethidium bromide staining, and recorded using the FireReader (UVITEC, Cambridge, UK) gel documentation system. The size of each amplified fragment was estimated by loading 250 bp step-up DNA ladder (B. Genei, Bangalore, India) as standard. To test the reproducibility, the amplifications and electrophoresis were repeated twice.

Preparation of extracts and determination of antioxidant and free radical scavenging activities Leaves and flowers were obtained from the *ex vitro*- and *in vitro*-derived *N. arbor-tristis* NAET-08 plants, air dried under shade, then crushed to granular particles using grinder (K-10, Bajaj Electrical Ltd., Mumbai, India) and kept in an air-tight plastic jar at 25°C. To obtain aqueous extracts, 10 g of dried powder of each plant part were soaked in 1000 mL of double-distilled (dd) H_2O at 60°C for 36 h, under agitation in a water bath. These decoctions were centrifuged at 12000 x g for 15 min, and the supernatants were collected in amber bottles and dried at 45°C, using a rotary vacuum evaporator (RV-10; IKA® WERKE GmbH & Co. KG, Staufen, Germany). To obtain methanolic and ethyl acetate extracts, each crushed sample (20 g) was extracted with 2000 mL of methanol or ethyl acetate, respectively, using a Soxhlet extractor. The extracts were dried as explained above and kept in amber bottles at 4°C. The yield percentage (*w/w*) was calculated following Mishra *et al.* (2016). From each of these dried extract preparations, 25 mg mL^{-1} stock solutions were prepared and used for the determination of polyphenol and flavonoid content, total antioxidant activity (TAA) and ferric ion reducing antioxidant power (FRAP), following protocols of Gul *et al.* (2011). The total polyphenols and flavonoids were expressed as gallic acid equivalents (μg GAE mg^{-1} dry weight) and quercetin

equivalents (μg QE mg^{-1} dry weight) per mg dry weight, respectively. Similarly, TAA and FRAP were expressed as ascorbic acid equivalents (μg AAE mg^{-1} dry weight) per mg dry weight.

The free radical scavenging activities of the extracts were also assessed for 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), and superoxide radicals (Kakkar *et al.* 1984; Braca *et al.* 2002; Gul *et al.* 2011) and the radical scavenging activity of each extract was measured as inhibition percentage, by using the formula:

Radical scavenging activity $(\%) = [(A_0 - A_e)/A_0] \times 100$, where A_0 and A_e were absorbance measured at 517 nm (A_{517}) of the control and extract(s), respectively.

Estimation of bioactive metabolites Ursolic acid. The dried methanolic extract (10 g) was suspended in Milli-Q water and partitioned successively with ethyl acetate, n-butanol, and water (aqueous extract). The ethyl acetate fraction (2.3 g) was chromatographed over silica gel using a step gradient of petroleum ether (1000 ml; fraction-I), petroleum ether: Chloroform 1:1 (*v/v*; 2000 ml fraction-II), Chloroform (2000 ml; fraction-III), chloroform: methanol 9:1 (*v/v*; 2000 ml; fraction-IV) as described by Saini *et al.* (2014), and four different fractions were eluted. Fraction-IV was separated through 1515 Isocratic high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA), using Whatman® 0.45 μm membrane (Merck KGaA, Darmstadt, Germany) and 20 μL of injected volume was analyzed using a C-18, 4.6 × 2 0 mm, 5 μm particle size XTerra™ RP column (Waters Corporation, Milford, MA). The entire HPLC analysis was carried out at 25 ± 2°C, the eluate was observed at 210 nm, and ursolic acid content (*w/w*) was estimated by standard curve method.

Rengyolone and nyctanthoside. The dried methanolic extracts (9.2 g) were chromatographed on a (70 to 230 mesh) silica gel column and fractionated successively as described by Tuntiwachwuttikul *et al.* (2003), and fraction I (198 to 224 mg) was purified over silica gel by step gradient elution, which gave rise to the expected colorless oil (453 to 512 mg) identified as rengyolone. Fractions II and III were also purified, eluted, and chromatographed as described previously by Tuntiwachwuttikul *et al.* (2003), and 92 to 108 mg of a resin-like substance was obtained, which was identified as nyctanthoside. The content of rengyolone and nyctanthoside (*w/w*) was measured for each sample.

Arbortristoside-A. The dried methanolic extract (~ 10 g) of each sample was dissolved in ddH_2O and fractionated with diethyl ether, ethyl acetate, and n-butanol. The n-butanol fraction [yield: 45.42 to 48.75% (*w/w*)] of each sample was dried, ground with acetone, washed with 2 N HCl, and subsequently with hot ddH_2O at 60°C. Finally, the fraction crystallized using a solvent system of 1:1 chloroform:methanol, and a light yellow-colored crystalline powder (308 to 344 mg) was obtained, and validated as arbortristoside-A using spectral analysis (Mendham *et al.* 2003), and its solubility in dimethyl sulfoxide (DMSO).

Statistical analysis Visual observations were taken with respect to the effects of plant growth regulators, as well as pretreatment conditions, on the frequency of regeneration, number of shoots per explants, shoot length, percentage of rooting, and root length. All the experiments were repeated twice, with five replicas per experiment, in randomized methods, and the data were represented as mean ± standard error (SE). The data were subjected to one-way analysis of variance (ANOVA) and the arithmetic means were compared ($P = 0.05$) using Duncan's multiple range test (Harter 1960) and the statistical software package SPSS® version 20.0 (IBM, Armonk, NY). The data on bioactive metabolite content, antioxidant activity, and radical scavenging activities were also statistically analyzed in the same way, also using SPPS® version 20.0 (IBM, Armonk, NY). The half concentration of inhibition (IC_{50}) values were calculated from linear regression analysis using EXCEL add-in program ED50 plus version 1.0 (http://www.softlookup.com/display.asp? id=2972; Vargas 2000).

Results and Discussion

Effects of PGRs and growth additives on *in vitro* induction and multiplication of shoots In the present study, the nodal explants of *N. arbor-tristis* cultured on MS medium fortified with different combinations of BA, KIN, and NAA exhibited morphogenic responses after 2 to 4 wk of culture. The nodal explants embedded in MS medium supplemented with 10.0 μM BA and 2.5 μM NAA responded well, as evidenced by the emergence of multiple shoot buds around the preexisting lateral meristem of the explants by the end of the second week (Fig. 1*a*), followed by the proliferation of multiple apical shoots after 4 wk of culture (Fig. 1*b*). These shoot buds proliferated to give rise 6 to 8 shoots (average of 6.60 ± 0.75; Table 1) per explant at the end of the eighth week, and the average shoot length for the PGR combination of 10.0 μM BA and 2.5 μM NAA was 3.04 ± 0.10 cm (Table 1). This response might be due to the coupled effects of low concentration of auxin along with high concentration of cytokinin on *in vitro* shoot multiplication, as reported in *Rhinacanthus nasutus* (L.) Kurz (Cheruvathur *et al.* 2012), *Sida cordifolia* L. (Sivanesan and Jeong 2007), and *Trichodesma indicum* (L.) Lehm. (Mahesh and Jeyachandran 2013). This exogenous application of auxin in low concentration might be involved in the asymmetric distribution of auxins necessary for the acquirement of organogenic competence, subsequent entry of active meristems and its adjoining cells into the cell cycle, canalized cell division, and *de novo* initiation of shoot buds under culture conditions, which then differentiate into

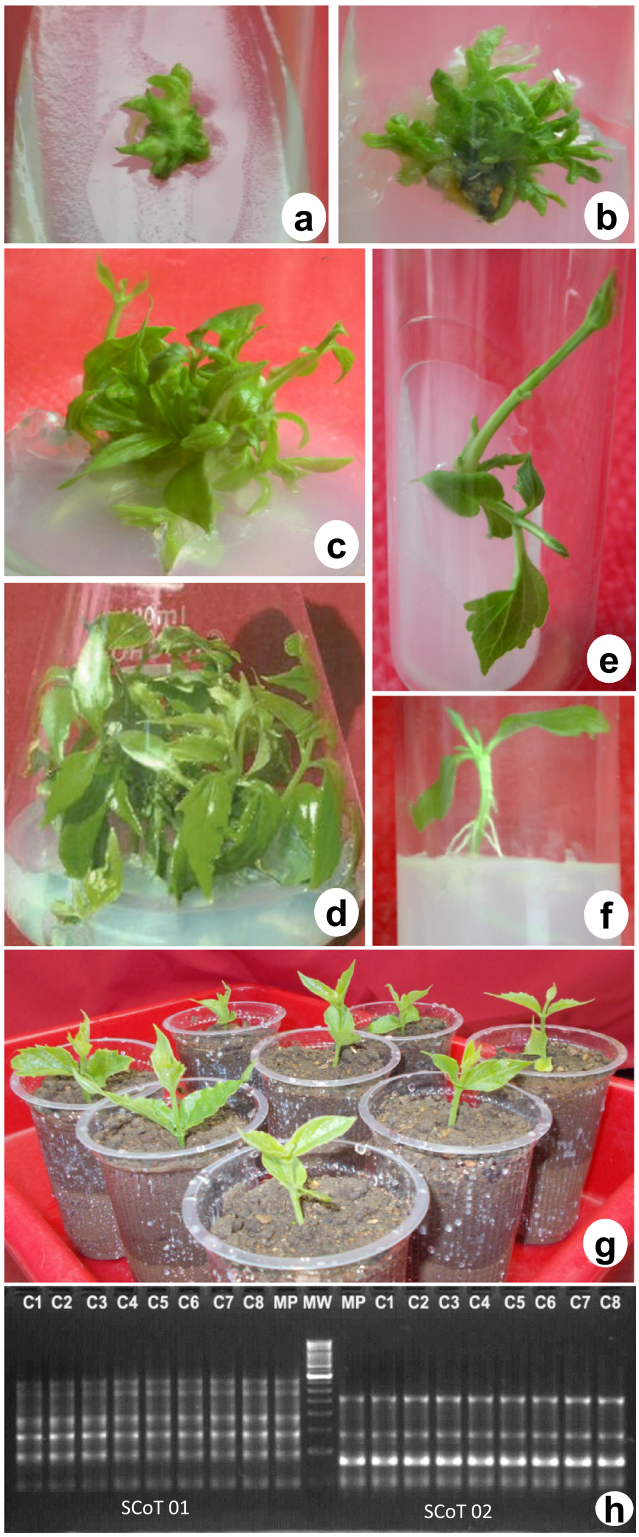

Figure 1. *In vitro* propagation in *Nyctanthes arbor-tristis*. (*a*) Emergence of shoot buds from the nodal explant on MS (Murashige and Skoog 1962) medium fortified with 10.0 µM 6-benzyladenine (BA) and 2.5 µM 1-naphthaleneacetic acid (NAA) at the end of 2 wk. (*b*) Proliferation of shoot buds showing multiple apical shoots at the end of 4 wk. (*c*) Proliferation of multiple shoots from the explant on MS medium fortified with 10.0 µM BA, 2.5 µM NAA, 45 µM adenine sulfate, 15 µM glutamine, and 10 µM proline at the end of 8 wk. (*d*) Elongation of shoots on the same medium after 8 wk. (*e*) Proliferation of multiple shoots from nodes pretreated with 100 µM *N*-phenyl-*N*′-benzothiazol-6-yl-urea (PBU) for 4 d and moved to on plant growth regulator (PGR) free MS medium at the end of 8 wk. (*f*) Emergence of roots from pretreated shoots on half strength MS medium without any PGR. (*g*) Acclimatization of shoots on plastic pots containing soil mix. (*h*) Genetic fidelity analysis of plantlets regenerated from PBU pretreated nodal explants using SCoT marker profiling (SCoT-1 and ScoT-2; *Lane-M*: 250 bp step-up ladder, MP-Donor plant; C1-C8: *in vitro* raised plantlets).

multiple shoots later on (Sugiyama 1999; Phillips 2004; Zhao *et al.* 2008). In an attempt to improve the multiplication rate, as well as shoot elongation, MS medium with optimized combination of PGRs was further enriched with 45 µM adenine sulfate, 15 µM glutamine, and 10 µM proline, which increased the number of shoots per explant (Fig. 1*c*) to 8.60 ± 0.63 (Table 2), but no significant effect on shoot elongation (Fig. 1*d*) was observed. The additive effects of adenine sulfate, glutamine, and proline on shoot multiplication have previously been reported in *Oryza sativa* and *Picrorhiza scrophularriflora* (Bantawa *et al.* 2009; Shahsavari 2011; Pawar *et al.* 2015). The addition of adenine sulfate might be

Table 1. Effects of 6-benzyladenine (*BA*), kinetin (*KIN*), and 1-naphthaleneacetic acid (*NAA*) on the shoot multiplication of *Nyctanthes arbor-tristis* L. cultured on MS (Murashige and Skoog 1962) medium for 8 wk

Plant growth regulators (μM)			Regeneration frequency (mean ± SE)*	Average number of shoots (mean ± SE)*	Shoot length (cm) (mean ± SE)*
BA	KIN	NAA			
0	0	0	0.0	0.0	0.0
2.5	–	–	55.40 ± 2.67 e	4.80 ± 0.37 bcd	0.78 ± 0.04 ab
5.0	–	–	61.60 ± 1.03 f	5.20 ± 0.37 bcde	1.54 ± 0.28 cdef
7.5	–	–	70.00 ± 0.71 g	5.60 ± 0.51 cde	1.38 ± 0.22 bcde
10.0	–	–	77.80 ± 1.20 h	6.20 ± 0.58 de	*3.36 ± 0.26* h
15.0	–	–	70.40 ± 0.75 g	5.60 ± 0.40 cde	2.18 ± 0.12 f
–	2.5	–	24.40 ± 1.17 a	2.80 ± 0.37 a	2.06 ± 0.21 ef
–	5.0	–	35.60 ± 1.17 b	3.80 ± 0.37 ab	1.14 ± 0.23 abcd
–	7.5	–	41.00 ± 1.10 c	4.20 ± 0.37 abc	1.96 ± 0.13 ef
–	10.0	–	40.40 ± 0.51 c	4.40 ± 0.51 bc	0.80 ± 0.09 ab
–	15.0		46.40 ± 1.17 d	4.80 ± 0.37 bcd	2.00 ± 0.06 abcd
10.0	–	2.5	*88.00 ± 1.10* i	*6.60 ± 0.75* e	3.04 ± 0.10 efg
10.0	–	5.0	85.40 ± 0.75 i	6.20 ± 0.80 de	2.28 ± 0.26 f
10.0	–	7.5	78.80 ± 1.02 h	6.20 ± 0.37 de	2.14 ± 0.60 f
10.0	–	10.0	72.00 ± 1.41 g	5.80 ± 0.37 cde	0.62 ± 0.02 a
–	15.0	2.5	54.00 ± 0.89 e	5.20 ± 0.37 bcde	1.64 ± 0.32 def
–	15.0	5.0	71.00 ± 1.00 g	5.40 ± 0.51 cde	0.60 ± 0.05 a
–	15.0	7.5	60.80 ± 1.02 f	5.60 ± 0.51 cde	0.88 ± 0.06 abc
–	15.0	10.0	52.60 ± 0.87 e	5.00 ± 0.45 bcde	0.72 ± 0.05 ab

*The means within a *column* followed by the *same letter* are not significantly different at $P = 0.05$, as per the Duncan's multiple range test analysis

impeding the degradation of cytokinins, either by feed-back inhibition or by competing with the metabolites involved in cytokinin anabolism (Van Staden *et al.* 2008), whereas the addition of glutamine and proline to the medium might have provided alternative nitrogen sources for maintaining a high shoot bud induction rate (Shahsavari 2011; Pawar *et al.* 2015).

To improve the efficiency of shoot multiplication, preconditioning of explants with non-purine phenyl urea derivatives with cytokinin activity, such as TDZ, CPPU, and PBU, have been utilized in many species. Pretreatment of axillary buds of *N. arbor-tristis* with 75 μM TDZ for 8 d and subsequent implantation in MS medium showed a twofold increase in the rate of shoot multiplication (Jahan *et al.* 2011). Considering the structural similarity of PBU with TDZ, in the present study the nodal explants were pretreated for 2 to 8 d either with liquid MS medium containing either 25 to 125 μM PBU, or with the previously optimized combination of PGRs and growth additives, 10.0 μM BA + 2.5 μM NAA + 45 μM adenine sulfate + 15 μM glutamine + 10 μM proline, serving as the control before implantation in PGR-free MS medium (Fig. 2). Pretreatment of nodal explants in liquid MS medium with PBU had a significant effect on morphogenic differentiation and shoot bud induction, which culminated in increased number of shoots per explant (Table 3; Fig. 2). Specifically, pretreatment of nodal explants in liquid MS medium with 100 μM PBU for 4 d elevated the shoot proliferation rate by producing the highest number shoots per explant (17.40 ± 1.02) with an average shoot length of 5.96 ± 0.08 cm (Table 3; Fig. 1*e*) which was an average increment of 7 additional shoots per explant as compared to the control (Fig. 2). This increased rate of shoot proliferation might be attributed to PBU-mediated altered cytokinin metabolism by inhibiting the activity of cytokinin oxidase, and accumulation of adenine cytokinins in the explant, as has been observed with TDZ (Jahan *et al.* 2011; Kumari *et al.* 2018). Similar effects of PBU were also reported in *Eucalyptus urophylla* S. T. Blake (Huang *et al.* 2010; Li *et al.* 2015), *Hyssopus officinalis* L. (Rolli *et al.* 2011), and *Capparis spinosa* L. (Carra *et al.* 2012). On comparison between the effect of preconditioning with 100 μm PBU for 4 d and 75 μM TDZ for 8 d (Jahan *et al.* 2011), the effect of TDZ on the rate of shoot proliferation was slightly superior in term of the number of shoots per explant by producing an average of 20.0 ± 1.15 shoots per explant. This difference might be attributed to either the differential response of different explants or different genotypes or different non-purine phenyl urea compound with cytokinin activity used in both the studies. Although these non-purine phenyl urea derivatives have been reported to have a positive effect on shoot proliferation (Malik *et al.* 2010; Rolli *et al.* 2011), in many instances of deleterious effects on shoot elongation, fasciation of shoots, and poor rooting have also been noticed (Huetteman and Preece 1993;

Table 2. Effect of adenine sulfate, glutamine, and proline on multiple shoot bud regeneration from nodal explants of *Nyctanthes arbor-tristis* L. cultured on optimized MS (Murashige and Skoog 1962) medium containing 10.0 µM 6-benzyladenine and 2.5 µM 1-naphthaleneacetic acid after 8 wk

Additive concentration (µM)			No. of shoot buds per explants (mean ± SE)*
Adenine sulfate	Glutamine	Proline	
Control			6.20 ± 0.37 abcde
15.0	–	–	5.60 ± 0.40 ab
30.0	–	–	5.60 ± 0.40 ab
45.0	–	–	5.80 ± 0.37 abc
60.0	–	–	6.80 ± 0.20 bcdef
–	7.5	–	6.40 ± 0.40 abcde
–	15.0	–	6.40 ± 0.40 abcde
–	22.5	–	6.80 ± 0.20 bcdef
–	30.0	–	7.00 ± 0.32 cdef
–	–	5.0	5.40 ± 0.25 a
–	–	10.0	5.60 ± 0.40 ab
–	–	15.0	5.60 ± 0.40 ab
–	–	20.0	6.00 ± 0.45 abcd
30.0	15.0	–	7.40 ± 0.25 ef
45.0	15.0	–	7.80 ± 0.37 f
60.0	15.0	–	7.20 ± 0.37 def
30.0	15.0	10.0	6.80 ± 0.37 bcdef
30.0	15.0	15.0	6.80 ± 0.37 bcdef
30.0	15.0	20.0	7.80 ± 0.37 f
45.0	15.0	10.0	8.60 ± 0.63 g
45.0	15.0	15.0	7.20 ± 0.37 def
45.0	15.0	20.0	6.80 ± 0.49 bcdef
60.0	15.0	10.0	6.20 ± 0.37 abcde
60.0	15.0	15.0	5.60 ± 0.25 ab
60.0	15.0	20.0	5.60 ± 0.45 abcd

*The means within a *column* followed by the *same letter* are not significantly different at $P = 0.05$, as per the Duncan's multiple range test analysis

Guo *et al.* 2011). In turn, these negative effects might be due to PBU-accumulation in the plant tissues, because of its inefficient degradation by cytokinin oxidase (Zatloukal *et al.* 2008; Podwyszyńska *et al.* 2014). However, in the present study, no such adverse effects of PBU pretreatment had been noticed among the *in vitro*-regenerated *N. arbor-tristis* plantlets.

***In vitro*-rooting and acclimatization** Well-elongated shoots, about 4 to 5 cm long, were separated from the originating clump and implanted in rooting medium containing half-strength MS salts fortified with either IBA or NAA (Table 4). No root induction was observed from the shoots implanted in the rooting medium, even after 2 wk of incubation. Thus, these preincubated shoots were transferred to half-strength MS medium without extraneous auxin, where after 3 to 4 d roots were induced from the cut ends, and in some cases from 1 cm above the cut end (Fig. 1*f*). The duration for complete root development was almost 3 to 4 wk. Preincubation of shoots with rooting medium containing 6 µM IBA for 1 wk, followed by implantation on auxin-free half-strength MS medium, showed the best response (93.00 ± 0.89%) for rooting, with an average development of 6.20 ± 0.49 roots per shoot, and an average root length of 1.62 ± 0.05 cm (Table 4).

Between the two auxins tested, preincubation with IBA showed its superiority in terms of root induction and root growth, compared to that of NAA (Table 4), and this might be due to IBA's presence in conjugated form, its stability, consistent weak auxin activity, and to IBA's insensitivity to auxin-degrading enzymes, as reported in studies with *Arabidopsis thaliana* and *Abutilon indicum* (Ludwig-Müller *et al.* 2005; Seth and Panigrahi 2018). However, the average root length varied from 0.66 to 1.62 cm, depending upon the type and concentration of auxins added to the medium. This might be due in part to the effect of IBA on the development of the root system by regulating primary root elongation and lateral root formation (Marquez *et al.* 2016). In the present study, almost 78% of *in vitro* regenerated plants had survived at the end of acclimatization (Fig. 1*g*), and this plant-to-soil establishment process took almost 4 wk. These *in vitro*-regenerated plantlets were morphologically similar to their respective *ex vitro*-grown donor plant, and produced flowers after 8 mo of plant establishment in soil.

Genetic fidelity of *in vitro*-regenerated plantlets Genetic variability is often observed during *in vitro*-propagation of many plant species and is attributed to organogenic induction driven by PGRs, culture environment, and epigenetic influences resulting in gene and ploidy mutations (Larkin and Scowcroft 1981; Kaeppler *et al.* 2000; Ramirez-Mosqueda and Iglesia-Andreau 2015). Use of PBU has been shown to induce 3.7% somaclonal variation during *in vitro*-propagation of *Citrus madurensis* Lour. *via* somatic embryogenesis (Siragusa *et al.* 2007). In light of these facts, genetic stability of *in vitro*-regenerated plantlets of *N. arbor-tristis* needed to be assessed, using DNA markers. Among different DNA markers, the SCoT marker analysis is quite simple and cost-effective, similar to random DNA markers; however, SCoT marker analysis exhibits increased stability and reliability due to the use of longer primers designed around the initiating codon (Collard and Mackill 2009; Agarwal *et al.* 2015). Moreover, these SCoT markers target the sequence flanking the start codon (Collard and Mackill 2009), which can provide further correlation with functional genes. In the present study, SCoT marker analysis using 10 primers produced consistently 33 amplified fragments (320 to 2405 bp; Table 5) among 39 regenerated plantlets and their donor plant (Fig. 1*h*; Table 5). This monomorphic banding pattern (Fig. 1*h*) demonstrated the lack of genetic variation among these *in vitro*-regenerated plantlets and their donor plant

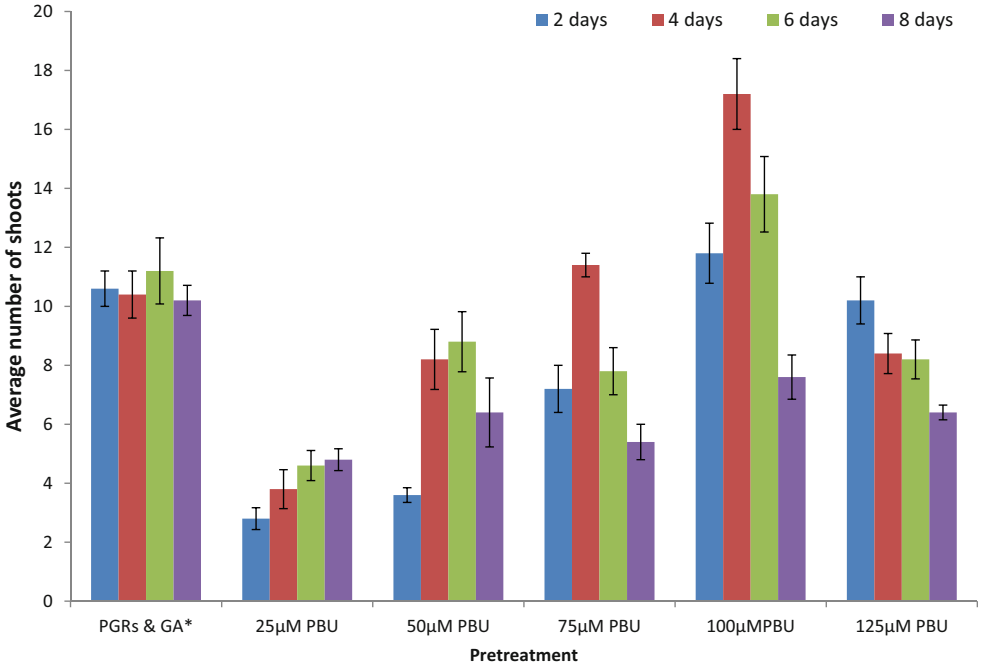

Figure 2. Graphical representation showing the effect of *N*-phenyl-*N*′-benzothiazol-6-yl-urea (PBU) pretreatment (dose and duration) on average number of shoots per nodal explant in *Nyctanthes arbor-tristis*.

Table 3. Effect of preconditioning *Nyctanthes arbor-tristis* L. node explants with different durations and concentrations of *PBU* and optimized growth regulator combination on multiple shoot bud regeneration after 8 wk culture

Preconditioning treatment of node explants	Treatment duration in d	% Regeneration[#] (mean ± SE)	No. of shoots per explants[#] (mean ± SE)	Shoot length in cm[#] (mean ± SE)
BA (10.0 µM) + NAA (2.5 µM) + AdS (45 µM) + glutamine (15 M) + proline (10 µM)	2	65.60 ± 0.51 ghi	10.60 ± 0.60 klm	5.68 ± 0.09 jkl
	4	73.40 ± 0.75 mno	10.40 ± 0.80 klm	5.80 ± 0.13 kl
	6	80.40 ± 0.68 pq	11.20 ± 1.12 lm	5.72 ± 0.1 kl
	8	68.00 ± 0.71 ij	10.20 ± 0.51 jklm	4.28 ± 0.23 fg
25 µM PBU	2	56.40 ± 0.51 bc	2.80 ± 0.37 a	2.34 ± 0.09 a
	4	60.40 ± 0.68 de	3.80 ± 0.66 abc	2.88 ± 0.12 b
	6	64.80 ± 1.39 gh	4.60 ± 0.51 abcd	4.48 ± 0.18 gh
	8	58.40 ± 0.51 cd	4.80 ± 0.37 abcde	3.08 ± 0.16 bc
50 µM PBU	2	72.40 ± 0.75 lmn	3.60 ± 0.25 ab	4.56 ± 0.05 gh
	4	76.40 ± 0.68 p	8.20 ± 1.02 ghijk	3.82 ± 0.09 de
	6	73.60 ± 1.03 mno	8.80 ± 1.02 hijk	2.46 ± 0.20 a
	8	68.60 ± 1.08 jk	6.40 ± 1.17 defgh	4.44 ± 0.23 g
75 µM PBU	2	71.80 ± 0.66 lm	7.20 ± 0.80 efghi	5.26 ± 0.10 ij
	4	74.60 ± 0.75 nop	11.40 ± 0.40 lm	3.46 ± 0.11 cd
	6	66.60 ± 0.40 hij	7.80 ± 0.80 fghij	5.62 ± 0.22 jkl
	8	58.20 ± 0.66 cd	5.40 ± 0.60 bcdef	3.92 ± 0.11 ef
100 µM PBU	2	70.60 ± 1.08 kl	11.80 ± 1.02 mn	5.54 ± 0.09 jkl
	4	82.20 ± 0.80 q	17.20 ± 1.20 p	5.96 ± 0.08 l
	6	76.20 ± 0.66 p	13.80 ± 1.28 no	5.86 ± 0.07 l
	8	63.80 ± 0.66 fg	7.60 ± 0.75 fghi	4.88 ± 0.12 hi
125 µM PBU	2	62.20 ± 0.66 ef	10.20 ± 0.80 jklm	4.52 ± 0.10 gh
	4	75.40 ± 1.40 op	8.40 ± 0.68 ghijk	5.78 ± 0.11 kl
	6	54.20 ± 0.66 b	8.20 ± 0.66 ghijk	5.38 ± 0.09 jk
	8	32.80 ± 1.02 a	6.40 ± 0.25 defgh	4.38 ± 0.07 g

[#] The means within a *column* followed by the *same letter* are not significantly different at $P = 0.05$, as per the Duncan's multiple range test analysis

BA 6-benzyladenine, *NAA* 1-naphthaleneacetic acid, *AdS* adenine sulfate, *PBU N*-phenyl-*N*′-benzothiazol-6-yl-urea

Table 4. Effect of auxin type and concentration on rooting percentage, root number, and root length of *Nyctanthes arbor-tristis* L. cultured on half-strength MS (Murashige and Skoog 1962) medium for 3 wk

Growth regulators concentration (μM)		% Rooting (mean ± SE)*	Number of roots (mean ± SE)*	Root length in cm (mean ± SE)*
IBA	NAA			
2.0	–	41.00 ± 1.41 b	2.80 ± 0.37 ab	0.74 ± 0.12 ab
4.0	–	66.20 ± 1.74 e	3.80 ± 0.37 bcd	0.94 ± 0.12 abc
6.0	–	93.00 ± 0.89 h	6.20 ± 0.49 e	1.62 ± 0.05 e
8.0	–	85.40 ± 0.87 g	4.20 ± 0.37 cd	1.10 ± 0.08 cd
10.0		79.60 ± 1.12 f	3.40 ± 0.24 ab	1.02 ± 0.18 bc
–	2.0	35.80 ± 0.66 a	2.60 ± 0.24 a	0.66 ± 0.07 a
–	4.0	41.60 ± 1.36 b	3.80 ± 0.37 bcd	0.98 ± 0.07 bc
–	6.0	50.20 ± 0.86 d	4.80 ± 0.37 d	1.36 ± 0.07 de
–	8.0	45.80 ± 1.02 c	3.8 ± 0.37 bcd	1.02 ± 0.07 bc
	10.0	46.20 ± 1.2 c	3.8 ± 0.24 bcd	1.10 ± 0.05 cd

*The means within a *column* followed by the *same letter* are not significantly different at $P = 0.05$ as per the Duncan's multiple range test analysis

IBA indole-3-butyric acid, *NAA* 1-naphthaleneacetic acid

of *N. arbor-tristis*, in consonance with their morphology, and offered additional authentication supporting the stability of this *in vitro* propagation protocol. Furthermore, SCoT marker analysis has been effectively used for the genetic homogeneity assessment of *in vitro*-regenerated plantlets in *H. isora*, *A. indicum*, and *A. maurorum* (Agarwal *et al.* 2015; Muthukumar *et al.* 2016; Seth *et al.* 2017).

Comparative evaluation of bioactive phytochemicals and antioxidant activity among leaf and flower extracts of *ex vitro*- and *in vitro*-grown plantlets *N. arbor-tristis* plant parts are the source of many important bioactive metabolites and have a wide range of biological and pharmacological activities (Rani *et al.* 2012; Agrawal and Pal 2013). The homogeneity and consistency of bioactive metabolite composition and accumulation in the target tissues of *in vitro*-raised plantlets should be similar to their *ex vitro*-grown donor plants as these plantlets will be the source of raw material for the isolation several therapeutic compounds. In view of these facts, the *in vitro*-regenerated plantlets and their plant parts produced in the current study were assessed for content of four important bioactive metabolites (ursolic acid, rengyolone, arbortristoside-A, and nyctanthoside), mostly used for the treatment of filaria and malaria (Tuntiwachwuttikul *et al.* 2003; Agarwal *et al.* 2013; Saini *et al.* 2014). The dried extracts under different solvents were assessed for yield percentage (*w/w*), which was 32.58% for methanolic, 20.36% for ethyl acetate, and 36.48% for aqueous extract, respectively. Ursolic acid was obtained only from leaf tissues of both the *ex vitro*- and *in vitro*-derived samples (Fig. 3*a*), whereas the

Table 5. Genetic fidelity assessment of *Nyctanthes arbor-tristis* L. *in vitro*-raised plantlets grown from 75 μM *N*-phenyl-*N*′-benzothiazol-6-yl-urea-pretreated nodal explants and their donor plant, using start codon targeted (*SCoT*) markers

Primer code	Primer sequence (5′–3′)	GC (%)	Number of fragments amplified	Range (bp)	Nature
SCoT-01	CAACAATGGCTACCACCA	50.00	6	485–1780	Monomorphic
SCoT-02	CAACAATGGCTACCACCC	55.56	4	430–1300	Monomorphic
SCoT-03	CAACAATGGCTACCACCG	55.56	2	650–965	Monomorphic
SCoT-04	CAACAATGGCTACCACCT	50.00	1	1025	Monomorphic
SCoT-05	CAACAATGGCTACCACGA	50.00	1	1200	Monomorphic
SCoT-06	CAACAATGGCTACCACGC	55.56	5	605–2405	Monomorphic
SCoT-07	CAACAATGGCTACCACGG	55.56	6	545–2030	Monomorphic
SCoT-08	CAACAATGGCTACCACGT	50.00	1	1555	Monomorphic
SCoT-09	CAACAATGGCTACCAGC	50.00	5	320–1590	Monomorphic
SCoT-10	CAACAATGGCTACCAGCC	55.56	2	1050–1590	Monomorphic
	Total		33	320–2405	Monomorphic

remaining three phytochemicals were isolated and quantified from both leaves and flowers (Fig. 3*b*). Leaves obtained from *in vitro*-propagated plantlets contained 0.79 ± 0.03 μg mg^{-1} ursolic acid, 1.95 ± 0.05 μg mg^{-1} rengyolone, 1.69 ± 0.03 μg mg^{-1} arbortristoside-A, and 1.08 ± 0.02 μg mg^{-1} nyctanthoside, whereas leaves of *ex vitro*-grown donor plants contained 0.78 ± 0.02 μg mg^{-1} ursolic acid, 1.84 ± 0.05 μg mg^{-1} rengyolone, 1.68 ± 0.04 μg mg^{-1} arbortristoside-A, and 1.13 ± 0.06 μg mg^{-1} nyctanthoside (Fig. 3*a*). Akin to leaf tissues, the flowers of *in vitro*-derived plantlets contained 2.63 ± 0.12 μg mg^{-1} rengyolone, 1.90 ± 0.01 μg mg^{-1} arbortristoside-A, and 1.43 ± 0.03 μg mg^{-1} nyctanthoside, whereas flowers of *ex vitro*-grown plants contained 2.51 ± 0.07 μg mg^{-1} rengyolone, 1.87 ± 0.05 μg mg^{-1} arbortristoside-A, and 1.41 ± 0.02 μg mg^{-1} nyctanthoside (Fig. 3*b*). By comparison, the respective bioactive metabolites accumulated in leaves and flowers of *in vitro*-regenerated plantlets were nearly equal to those of the *ex vitro*-grown donor plants for ursolic acid, arbortristoside-A, and nyctanthoside content (Fig. 3*a*, *b*). However, the rengyolone content in the leaves and flowers of *in vitro*-regenerated plantlets was noticeably higher, compared to donor plants (Fig. 3*a*). This variation might be attributed to the influences of the PGRs used, tissue composition of explants, and culture environment, which probably necessitated the production and accumulation of more rengyolone, as has been reported for different bioactive metabolites in several medicinal species including *Hypericum hirsutum* L, *H. maculatum* Crantz., *Agastache rugosa* O. Kuntze, *Musa accuminata* L., and *Aloe arborescens* L. (Coste *et al.* 2011; Zielinska *et al.* 2011; Adeyemi *et al.* 2012; Amoo and Van Staden 2013).

Antioxidant activity The harmful effects of free radicals and reactive oxygen species can be alleviated by antioxidant substances, preferably from natural sources, to prevent toxicity (Li *et al.* 2014). Polyphenols and flavonoids have been reported to be associated with such antioxidant activities (Gul *et al.* 2011; Riaz *et al.* 2014). A wide array of phytochemicals with proven antioxidant activities (Dasgupta and De 2007; Rathee *et al.* 2007; Khanapur *et al.* 2014; Mishra *et al.* 2016) has been identified in *N. arbor-tristis* plant parts. Thus, in the present study,

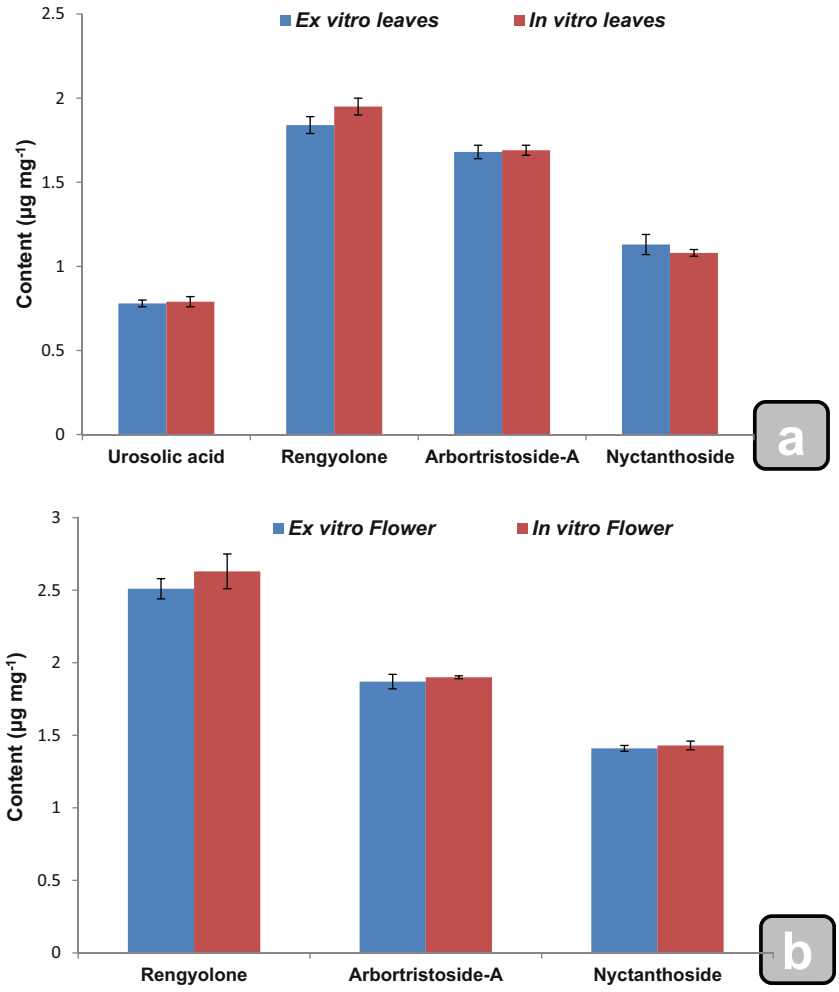

Figure 3. Comparison of four bioactive metabolites content in the leaves (*a*) and flowers (*b*) of *Nyctanthes arbor-tristis* plants raised *in vitro* as well as *ex vitro*.

three different extracts of leaves and flowers from *ex vitro*- and *in vitro*-grown plants were compared for total polyphenols and flavonoids content, as well as TAA, FRAP, and radical scavenging activities. As reported previously, both flower and leaf extracts of *N. arbor-tristis* show quite promising amounts of natural bioactive metabolites contributing to antioxidant and radical scavenging activities (Dasgupta and De 2007; Rathee *et al.* 2007; Mishra *et al.* 2016). Results obtained in the present study showed that total antioxidant activity of leaf extracts was higher than those of corresponding flower extracts (Fig. 4*c*), and a similar trend was noticed for polyphenols content (Fig. 4*a*), flavonoids content (Fig. 4*b*), and ferric ion reducing antioxidant power (Fig. 4*d*) among the extracts (Table 6). Similar canonical relationships between total polyphenol content and TAA have been established in a number of other medicinal plants including *Abelmoschus moschatus* Medik. L. and *Fraxinus rhynchophylla* Hance (Li *et al.* 2008; Gul *et al.* 2011). The antioxidant activity of different plant-based extracts is usually correlated directly to its reducing capacity, and the FRAP assays (Fig. 4*d*) conducted here substantiated prominent antioxidant activity (Fig. 4*c*) of leaf and flower extracts of *N. arbor-tristis* during this study (Table 6). Similar FRAP assays have been performed to evaluate antioxidant activity of flavonoid-rich tissue extracts in different species (Luximon-Ramma *et al.* 2002; Gul *et al.* 2011). In the present study, the leaf extracts showed positive correlation between flavonoids content (Fig. 4*b*) and ferric ion reducing power (Fig. 4*d*); when compared to the flower extracts, the leaf extracts showed higher flavonoids content, as well as elevated ferric ion reducing ability (Table 6; Fig. 4*b*, *d*). In the comparisons between *in vitro*- and *ex vitro*-raised plant parts shown in Table 6, the methanolic and ethyl acetate leaf extracts of *in vitro*-regenerated plants showed higher flavonoid content (149.00 ± 2.31 QE μg mg^{-1}; Fig. 4*b*) and higher TAA (35.33 ± 0.88 AAE μg mg^{-1}; Fig. 4*c*), respectively. Similarly, methanolic flower extracts of *in vitro*-raised plants also showed little higher content of polyphenols (156.33 ± 4.33 GAE μg mg^{-1}) and TAA (25.0 ± 1.16 AAE μg mg^{-1}), when compared to their *ex vitro*-raised counterpart (Table 6; Fig. 4). The superiority of the oxidative potential of methanolic and ethyl acetate

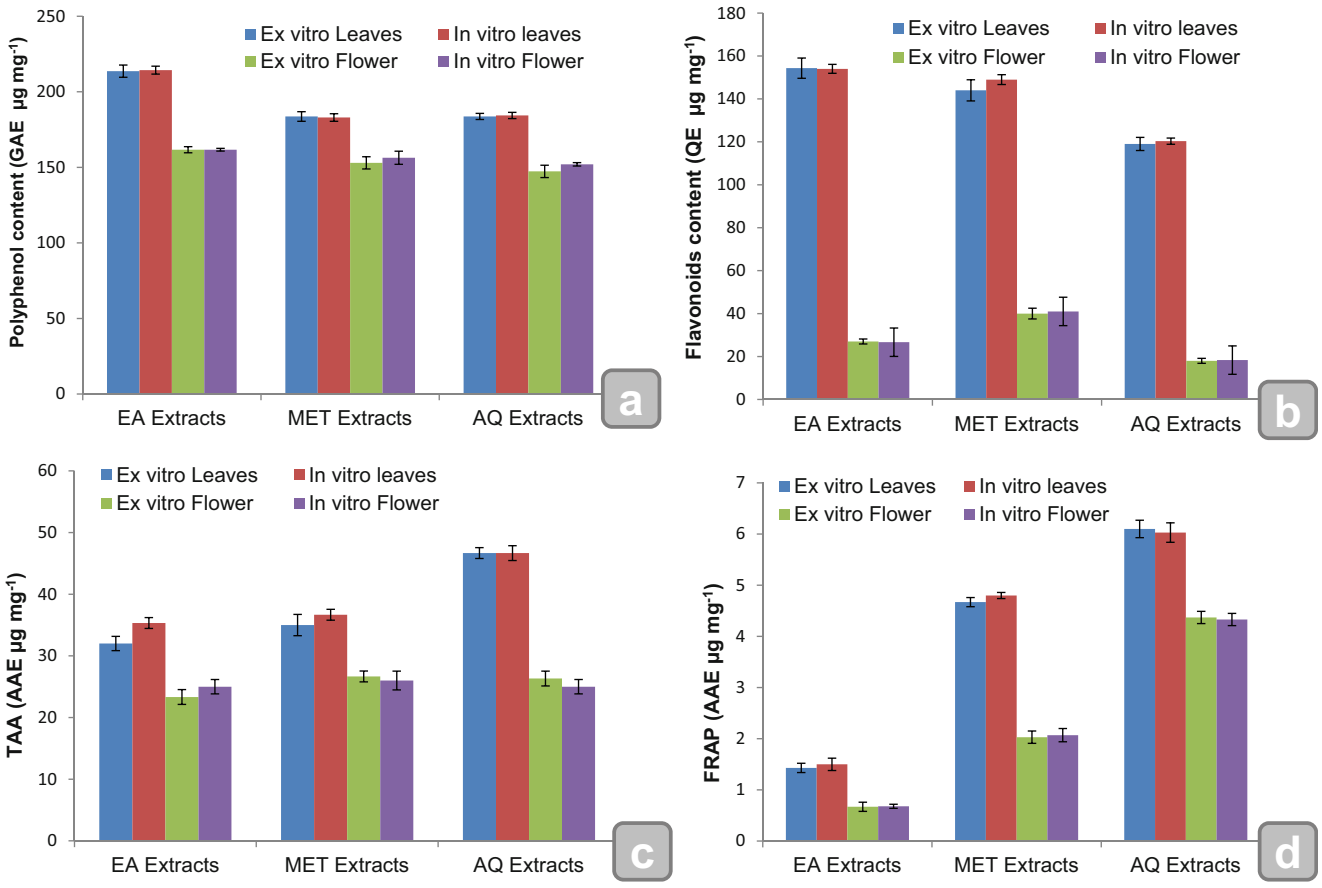

Figure 4. Comparative evaluation of antioxidant rich phytochemicals [(*a*) total phenol content and (*b*) total flavonoids content] and antioxidant potential [(*c*) total antioxidant activity and (*d*) ferric ion reducing power] of different extracts obtained from *Nyctanthes arbor-tristis* plants raised *in vitro* as well as *ex vitro*.

Table 6. Comparative evaluation of polyphenols, flavonoids, total antioxidant activity, and ferric ion reducing antioxidant power in *Nyctanthes arbor-tristis* L. leaves and flowers obtained from *in vitro* and *ex vitro*-grown plantlets

Phytochemicals (μg mg^{-1})#	Leaf extracts						Flower extracts					
	Ethyl acetate		Methanolic		Aqueous		Ethyl acetate		Methanolic		Aqueous	
	Ex vitro	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*
Polyphenols[p]	213.67 ± 4.05 f	214.33 ± 2.60 f	183.67 ± 3.18 d	183.00 ± 2.52 d	183.67 ± 2.03 e	184.33 ± 2.03 e	161.67 ± 2.03 d	161.67 ± 0.88 d	153.00 ± 4.04 b	156.33 ± 4.33 c	147.33 ± 4.06 a	152.00 ± 1.16 ab
Flavonoids[q]	154.33 ± 4.70 g	154.00 ± 2.08 g	144.00 ± 4.93 e	149.00 ± 2.31 f	119.00 ± 3.06 d	120.33 ± 1.45 d	27.00 ± 1.16 b	26.67 ± 0.88 b	40.00 ± 2.52 c	41.00 ± 1.53 c	18.00 ± 1.16 a	18.33 ± 0.33 a
TAA[r]	32.00 ± 1.16 c	35.33 ± 0.88 d	35.00 ± 1.73 d	36.67 ± 0.88 d	46.67 ± 0.88 e	46.67 ± 1.20 e	23.33 ± 1.20 a	25.00 ± 1.16 b	26.67 ± 0.88 b	26.00 ± 1.53 b	26.33 ± 1.20 b	25.00 ± 1.16 b
FRAP[s]	1.43 ± 0.09 b	1.50 ± 0.12 b	4.67 ± 0.09 e	4.80 ± 0.06 e	6.1 ± 0.17 f	6.03 ± 0.19 f	0.67 ± 0.89 a	0.68 ± 0.04 a	2.03 ± 0.12 c	2.07 ± 0.13 c	4.37 ± 0.12 d	4.33 ± 0.12 d

TAA total antioxidant activity; *FRAP* ferric ion reducing antioxidant power

*The means within a *row* followed by the *same letter* are not significantly different at $P = 0.05$, as per the Duncan's multiple range test analysis

Phytochemicals expressed as equivalents μg mg^{-1} dry weight for p, gallic acid; q, quercetin; r & s, ascorbic acid

Table 7. Comparative evaluation of half concentration of inhibition (IC_{50}) values* of ethyl acetate, methanolic, and aqueous extracts of *Nyctanthes arbor-tristis* L. leaves and flowers obtained from *in vitro* and *ex vitro*-grown plantlets on different free radicals tested.

Radicals tested	Leaf extracts						Flower extracts						Standard#
	Ethyl acetate		Methanolic		Aqueous		Ethyl acetate		Methanolic		Aqueous		
	Ex vitro	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	
DPPH	491.68 ± 1.09 e	499.91 ± 1.49 f	360.1 ± 5.82 c	346.09 ± 1.25 a	355.17 ± 2.09 bc	353.74 ± 3.15 b	418.99 ± 3.22 d	415.73 ± 2.98 d	360.77 ± 1.85 c	359.14 ± 1.37 c	508.93 ± 6.79 g	506.74 ± 1.68 g	21.17 ± 0.64 A
H_2O_2	571.31 ± 5.89 c	569.20 ± 3.89 hi	451.01 ± 0.70 d	442.17 ± 1.41 c	421.23 ± 1.58 b	413.16 ± 1.08 a	506.72 ± 2.88 f	566.28 ± 6.39 h	513.91 ± 2.86 g	499.57 ± 3.31 e	616.29 ± 4.28 k	593.01 ± 3.24 j	64.15 ± 1.2 B
SOR	471.93 ± 2.25 f	486.58 ± 0.45 g	356.62 ± 2.09 c	333.30 ± 2.57 b	317.81 ± 4.41 a	317.13 ± 3.52 a	1844.66 ± 21.5 k	1650.89 ± 20.7 j	446.21 ± 1.91 e	402.48 ± 4.21 d	1140.08 ± 4.72 i	1034.05 ± 12.7 h	46.80 ± 0.4 C

DPPH 1,1-diphenyl-2-picrylhydrazyl, *SOR* superoxide radicals

*The means within a *row* followed by the *same letter* the same letter are not significantly different at $P = 0.05$, as per the Duncan's multiple range test analysis

Standard *A* and *B*, ascorbic acid; standard *C*, quercetin

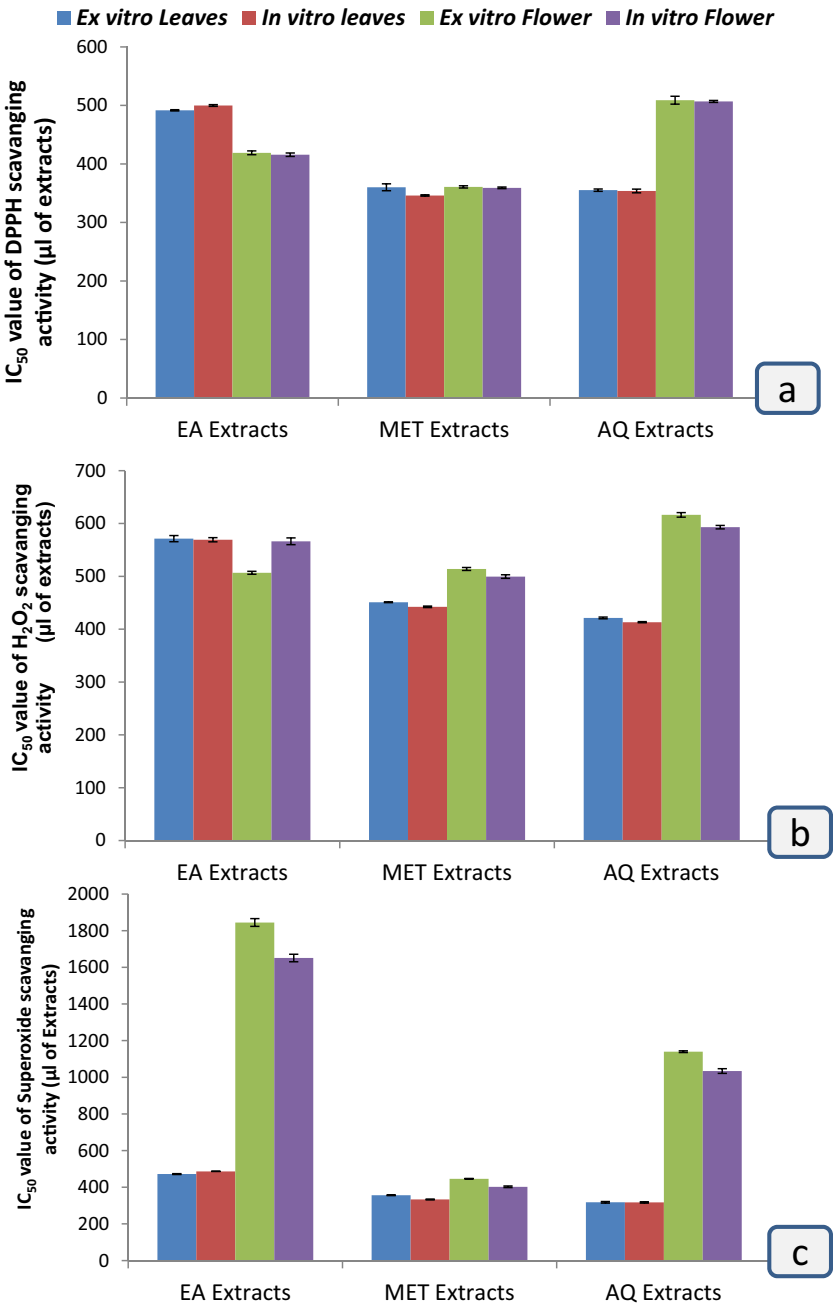

Figure 5. Comparative evaluation of free radicals [(*a*) 1,1-diphenyl-2-picrylhydrazyl (DPPH), (*b*) hydrogen peroxide (H_2O_2), (c) superoxide] scavenging ability (in term of IC_{50} value) of leaf and flower extracts obtained from *Nyctanthes arbor-tristis* plants raised *in vitro* as well as *ex vitro*.

extract of leave and flower, of *in vitro*-regenerated plant tissues might be due to the influence of PBU on the endogenous PGRs, as well as induced expression of genes involved in the biosynthesis of antioxidant-rich secondary metabolites and phytochemicals (Amoo and Van Staden 2013; Amoo *et al.* 2013). On the other hand, aqueous leaf and flower extracts of *ex vitro*-grown donor plant showed marginally higher, if statistically similar, FRAP and TAA activity (Table 6; Fig. 4*c*, *d*). This observed variation in total antioxidant activities among different extracts might also be attributed to solvents influencing either tissue-specific solubility of phytomolecules or environmental stresses encountered during *ex vitro*-growth (Masondo *et al.* 2015).

Radical scavenging activity The leaf and flower extracts of *N. arbor-tristis* were evaluated for their ability to scavenge DPPH, H_2O_2, and superoxide radicals in a concentration-dependent manner (Table 7; Fig. 5) in the current study, recognizing that observed variation depends upon the type of tissues and solvents, as reported earlier (Dasgupta and De 2007; Rathee *et al.* 2007; Mishra *et al.* 2016). Similar to

content of bioactive metabolites and antioxidant potential, radical scavenging activity of the extracts obtained from *in vitro*-regenerated plants were *on a par* with their respective counterpart from the donor plant, barring a few exceptions (Table 7; Fig. 5): the DPPH radical scavenging activity of analogous extracts of *ex vitro*- and *in vitro*-grown leaves and flowers were almost identical in terms of IC_{50} value, barring ethyl acetate and methanolic leaf extract (Fig. 5*a*). Ethyl acetate flower extracts showed superior DPPH radical quenching activity (as evidenced by lower IC_{50} values) in comparison to that of leaf extracts, regardless of plant origin (Fig. 5*a*), whereas aqueous leaf extracts revealed better DPPH scavenging activity than flower extracts (Fig. 5*a*). Contrasting to this, leaf extracts showed better H_2O_2 (Fig. 5*b*) and superoxide radical scavenging (Fig. 5*c*) activity than flower extracts (Table 7), for all categories, except for H_2O_2-scavanging activity of ethyl acetate-extracted leaves. In most cases both the *in vitro* and *ex vitro* leaf extracts showed equivalent radical scavenging activities in terms of their IC_{50} values, while *in vitro* flower extracts showed superiority over *ex vitro* flower extracts (Fig. 5). However, these variations might also have resulted due to either varying distribution of active components in different tissues, age of *in vitro*-regenerated plantlets grown in an *ex vitro*-environment, and fractionation during extraction, or even stresses levied during their growth, of the plants grown either *in vitro* or *ex vitro* (Malik *et al.* 2010; Riaz *et al.* 2014; Masondo *et al.* 2015).

Conclusion The present study established an effective and alternative protocol for the micropropagation of *N. arbor-tristis* plants from PBU-treated nodal explants *via* organogenesis, where genetic stability of regenerated plantlets was affirmed by SCoT marker profiling, and by homogenous content of four sampled therapeutical metabolites, antioxidant potential, and equivalent radical scavenging activities. The presented propagation procedure should be of immense use for large-scale, clonal multiplication of *N. arbor-tristis in vitro* to meet the raw material demands of both the herbal industries and for esthetic purposes, as well as for the *ex-situ* conservation of *N. arbor-tristis* genotypes.

Author's contribution JP conceived the project, designed the experiment, interpreted the results, and contributed to the writing of the manuscript; SCR performed the experiments, analyzed and interpreted the data, and contributed to the writing of the manuscript; SS, SKM, and PKY contributed towards the analysis of antioxidant activity and estimation of biomolecule content. AKG contributed towards critical evaluation of the findings and writing of the manuscript. All the authors have contributed towards the final version of the manuscript by writing and editing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals; hence, no ethical approval has been obtained from the concerned committee.

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