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RESEARCH NOTE

Impact of silver nanoparticles on the micropropagation of *Hybanthus enneaspermus* and assessment of genetic fidelity using RAPD and SCoT markers

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Abstract
A prompt and dynamic clonal propagation system was developed for *Hybanthus enneaspermus* a medicinally significant plant using shoot tip as explants. Multiple shoots were induced on Murashige and Skoog (MS) medium invigorated with different levels of 6-benzylaminopurine (BAP), kinetin (Kin) and thidiazuron (TDZ) and AgNPs. The optimum concentration of BAP (1.5 mg L^{-1}) with AgNPs (3.0 mg L^{-1}) exhibited a determined response of 90.66% and created a maximum of 77.23 shoots with a maximum shoot length of 3.72 cm after 4 weeks. The vigorous shoots were embedded on an MS medium modified with optimum concentration of IBA 1.0 mg L^{-1} and AgNPs 1.0 mg L^{-1}, which engendered the highest number of roots (9.65) with an average root length of 4.77 cm, with the response of 87%. Further, rooted plantlets were efficiently acclimatized in the soil. In addition, genetic homogeneity analysis was performed using RAPD and SCoT molecular marker, which revealed that the regenerated plants were genetically uniform and no detectable genetic difference was observed when compared to the mother plant. This efficient micropropagation protocol provides a new way for targeted gene editing for functional analysis of genes in *Hybanthus enneaspermus*.

Key message
The green synthesized AgNPs increased the regeneration frequency and there is no genetic variation among the invitro regenerated plants.

Keywords AgNPs · Shoot tip · Cytokinin · Shoot production · BAP

Abbreviations

AgNPs	Silver nanoparticles
PCR	Polymerase chain reaction
SCoT	Start codon targeted polymorphism
RAPD	Randomly amplified polymorphic DNA
FTIR	Fourier transform infrared
SEM	Scanning electron microscope

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Medicinal and aromatic plants are a good source of phytocompounds and play a significant role in the world health system. *Hybanthus enneaspermus* is traditionally used for the treatment of various ailments such as urinary infections, strangury, inflammation, leucorrhoea, asthma, demulcent, sterility, and dysuria (Patel et al. 2013). The plant contains various phytochemical constituents like isoaborinol, peptide alkaloid, aurantiamide acetate, sitosterol, L-Dopa, anthraquinones, diosgenin, and triterpene (Sathish et al. 2019).

In vitro regeneration of *H. enneaspermus* has been previously described by some workers by employing various kinds of plant parts including leaf, stem, node, and shoot tip

(Sivanandhan et al. 2015; Sudharson et al. 2014; Shekhawat and Manokari 2018), but were not effective in producing a greater number of shoots. To achieve higher transformation efficiency, an enhanced in vitro regeneration protocol with the potential to generate increased shoots is, therefore, a prerequisite.

Silver nanoparticles (AgNPs) are considered to be efficient in inhibiting ethylene activity due to their physiochemical properties and increased surface-to-volume ratio (Sarmast et al. 2015). Earlier studies demonstrated that supplementing silver ions and AgNPs in the culture medium rendered the explants healthier as compared to the ones grown in the control medium (Timoteo et al. 2019). Numerous PCR-based molecular markers play pivotal roles in the genetic stability analysis and among them, the random amplified polymorphic (RAPD, SCoT) marker has been extensively used (Ajithan et al. 2019). Hence the present investigation was aimed to establish an improved regeneration system and production of genetically stable *H. enneaspermus* plants that will be deployed for germplasm conservation and rapid multiplication.

AgNPs were synthesized by bioreduction of silver nitrate using *H.enneaspermus* leaf extract as described by Manickavasagam et al. (2019). The biosynthesized nanoparticles were categorized by UV-Visible spectroscopy (Cyber lab-100 spectrophotometer), Fourier transform infrared (FTIR), XRD, and scanning electron microscope (SEM) coupled with energy dispersive X-ray (EDX) analysis. Shoot tip explants taken from field-grown plants were surface sterilized (Sathish et al. 2019) and cultured on MS (Murashige and Skoog 1962) medium incorporated with various doses of (0.5–2 mg L^{-1}) benzyl amino purine (BAP), (0.5–2 mg L^{-1}) kinetin (Kin) and (0.5–2 mg L^{-1}) thidiazuron (TDZ). Explants placed on MS medium devoid of phytohormones maintained as control. After 6 weeks, the frequency of explant response, the number of shoots per explant, and the length of the shoots were documented.

To systematize the influence of AgNPs on multiple shoot production, shoot tip explants were placed on optimum concentration of BAP with different concentrations of AgNPs (1–5 mg L^{-1}) to enhance the multiple shoot production. Explants grown in MS medium with BAP devoid of AgNPs were maintained as control. The elongated shoots were excised individually and placed on MS medium with a diverse concentration of IBA, NAA (0.5–2 mg L^{-1}), and AgNPs (0.5–2 mg L^{-1}) to optimize root induction. Culture inoculated on hormone-free MS media failed to induce roots. After 4 weeks, the response of rooting, number of roots per shoot, and length of the root were measured. All the cultures were incubated at 25 ± 2°C under a 16/8 h photoperiod with 50 μ mol m^{-2} s^{-1}exposed by cool-white fluorescent tubes (Philips, India).

The well-rooted plants were taken out of the rooting medium after 4 weeks and placed in disposable cups comprising sterilized soil. The genomic DNA was extracted using the CTAB method from young leaves (200 mg) of in vitro regenerated plants and mother plants. DNA isolation and clonal fidelity analysis were carried out using 9 RAPD and 8 SCoT markers described by Vasudevan et al. (2017). The annealing temperature for RAPD (50–60 °C) and SCoT (50–60 °C) were used. Extracted DNA was then analyzed qualitatively and quantitatively using UV absorbance at 260/280 nm and loaded on a 0.8% agarose gel (w/v) to visualize DNA using the Gel Documentation system (UVITEC, Cambridge, UK). PCR reactions and program conditions for RAPD and SCoT were performed as defined by Vasudevan et al. (2017). PCR products were analyzed in a 1.0% agarose gel (w/v) (HiMedia, Mumbai, India) and visualized under a Gel Documentation system.

All the experiments were carried out in a randomized design in this research. The experiments were performed thrice with three replicates. Duncan's multiple range test (DMRT) was used to analyze the obtained data, and the significance level was set at 5%. The data were all presented as mean ± standard error.

The bio-reduction of silver ions was confirmed through the color changes in the reaction mixture from light brown to dark brown (Fig. 1a). The materialization of color changes in the solution was caused by surface plasmon excitation and the same has been reflected in UV–Vis spectrum analysis at 420 nm, which is the notable evidence for the formation of silver nanoparticles (Fig. 1b). FTIR spectra of AgNPs revealed various peaks which exhibit the complex nature of phytomolecules present in the extract (Fig. 1c). The spectra peaks appearing at 3410.55, 2927.34, 1613.82, 1098.60, and 619.76 were attributed to O–H band, C–H stretching, N–H stretching, C–O stretching and C–H bonding of aromatic compounds respectively. The XRD observed with 20° at 38.12 44.30, 64.45, 77.41, and 81.55 which are in agreement with the (111), (200), (220), and (311) reflects the face-centered cubic crystalline structure of metallic nanoparticles respectively (Fig. 1d). The SEM analysis indicated that the morphology of the nanosilver structures is spherical in shape and size ranging between 5 and 100 nm (Fig. 1e). The EDX analysis proved the presence of silver in the nanoparticles. In the range of 3.0–4.0 keV, the EDX spectrum displays a clear metallic silver signal (Fig. 1f). Earlier similar kind of characterization results was reported in one of our work (Manickavasagam et al. 2019).

To induce the multiple shoots from shoot tips, the explants were grown on an MS medium comprising diverse doses of cytokinins such as BAP, TDZ, and Kin (Fig. 2a). The explants grown on MS medium lacked hormones and did not produce shoots. However, explants grown on MS medium augmented with diverse cytokinins such as BAP,

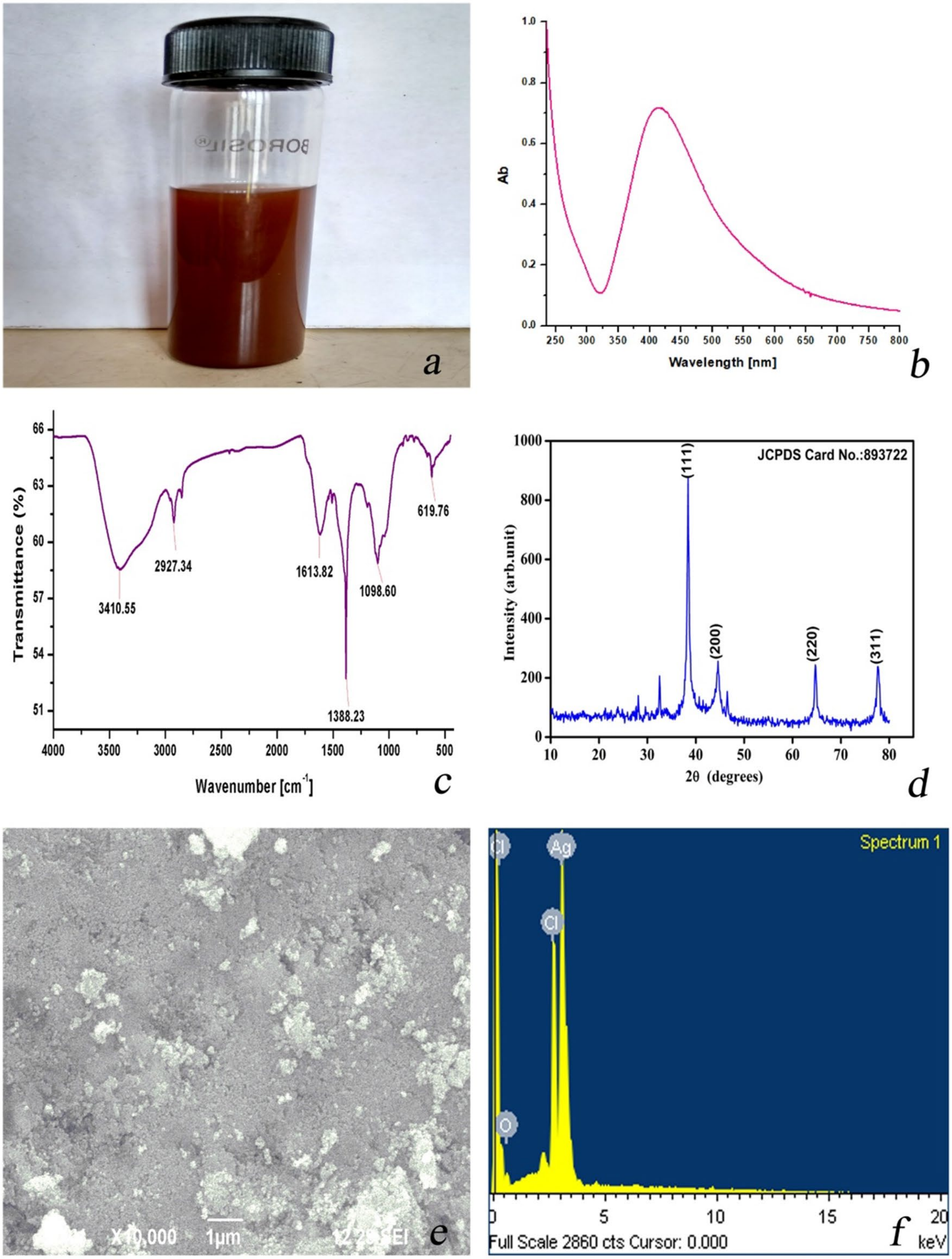

Fig. 1 AgNPs synthesis and characterization from *H. enneaspermus* plant extracts. **a** Green synthesized AgNPs, **b** UV spectrum analysis of AgNPs, **c** FTIR spectrum of AgNPs, **d** XRD pattern of synthesized AgNPs, **e** SEM image of AgNP, **f** EDX spectrum of AgNPs

TDZ, and Kin at various levels (0.5–2.5 mg L^{-1}) induced multiple shoots with various responses (Supplementary Table 1). Among the various cytokinins examined, BAP 1.5 mg L^{-1} displayed the highest response (70.66%), the number of shoots per explant (53.37) with shoot length (2.66 cm) after 6 weeks of culture. Kin 1.0 mg L^{-1} produced

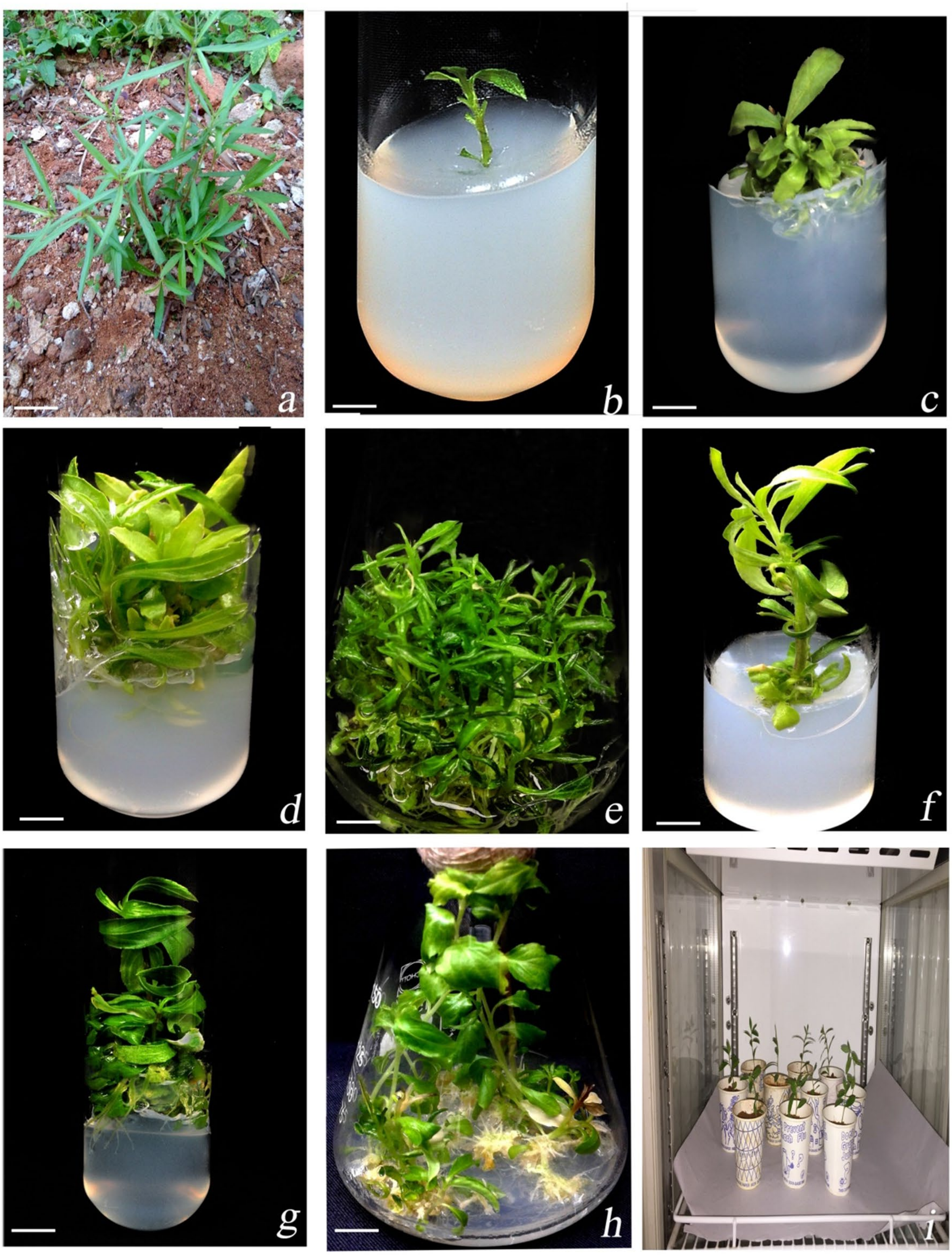

Fig. 2 The effect of BAP and AgNPs on multiple shoot production in *H.enneaspermus*. **a** Field grown plant, **b** Shoot tip explant inoculated on MS medium fortified with BAP 1.5 mg L^{-1} and AgNPs 3.0 mg L^{-1} (1 week old culture), **c** Initiation of shoots on MS medium containing BAP 1.5 mg L^{-1} and AgNPs 3.0 mg L^{-1} (2 week old culture), **d** Multiple shoots induction (3 weeks old culture), **e** Proliferation of multiple shoots (6 weeks old culture), **f** shoot elongation, **g**, **h** Rooting of elongated shoot in MS medium containing IBA 1.0 mg L^{-1} and AgNPs 1.0 mg L^{-1}, (2 week old culture) **i** Hardened plants in the paper cups (3 weeks old)

(27.50) shoots per explants with shoot length of 1.04 cm and TDZ 1.5 mg L^{-1} generated (34.64) shoots per explants with shoot length of 1.62 cm after 6 weeks. The regeneration response and the number of shoots were suppressed by higher BAP concentration levels. The influence of BAP on multiple shoot production from shoot tip explants has been widely documented in various medicinal plants (Upadhyay et al. 2015; Yanthan et al. 2017). Premkumar et al. (2013) reported that *H.enneaspermus* leaf explants reared on MS medium comprising IAA 5.71 µM along with Kn 4.64 µM and BAP 4.44 µM induced 52.3 shoots per explants. The cytokinin type of BAP has a notable effect on shoot induction and is relatively more stable when compared to other cytokinins.

To achieve a higher number of multiple shoots, AgNPs at different concentrations were fortified in an MS medium containing an ideal concentration of BAP 1.5 mg L^{-1} (Fig. 2b, c). Inclusion of 3 mg L^{-1} AgNPs with an optimal concentration of BAP in the MS medium resulted in 90.66% of shoot induction and produced 77.23 shoots with a shoot length of approximately a maximum of 3.72 cm per explants (Supplementary Table 2; Fig. 2d). The positive outcomes could be ascribed to the inhibitory influence of AgNPs on ethylene production (Kim et al. 2017; Sarmast et al. 2015) demonstrated that the amalgamation of AgNPs in the culture medium not only controlled the bacterial contamination, it also relatively enhanced the growth and number of shoots when compared to explants grown on AgNPs free culture medium. Similar to our results, the effect of AgNPs on multiple shoot induction was reported in *Swertia chirata* (Saha and Dutta Gupta 2018). Recently Jadczak et al. (2019) and Timoteo et al. (2019) proved the efficiency of silver nanoparticles on micropropagation of *Lavandula angustifolia* and *Campomanesia rufa* respectively. Aghdaei et al. (2012) reported that stem explants cultured on MS medium amended with AgNPs 10 mg L^{-1}, IAA 0.1 mg L^{-1}, and BAP 2.5 mg L^{-1} exhibited higher response and shoot induction in *Tecomella undulate*. As the AgNPs used in this experiment were prepared from the same *H.enneaspermus* plant, it may be speculated that the phyto components encapping the nanoparticles have added a benefit of being biocompatible and create a zone of zero contamination from the biological perspective of view when administered as foreign materials to the shoot tissue cultures. However, the exact mechanism of AgNPs on in vitro regeneration is still highly debatable further investigation need to be emphasized. Application of BAP (1.5 mg L^{-1}) and AgNPs (3.0 mg L^{-1}) in the MS medium promoted the highest shoot induction frequency in the same medium is accompanied by shoot elongation (Fig. 2e, f). The shoots were elongated in shoot induction medium, which reduced the time duration when compared to standard elongation steps. Sivanandhan et al. (2015) proved that the regenerated shoots of *H. enneaspermus* were elongated in the shoot induction medium.

The present research explores the well-developed healthy shoots were excised and cultured on an MS medium augmented with various concentrations (0.5-2.0 mg L^{-1}) of IBA, NAA, and AgNPs. All the treatments favoured root induction, but the percentage of response differed between the tested auxins and AgNPs (Supplementary Tables 3 and 4). The highest percentage of rooting (87%) and highest root number (9.65) with an average root length (4.77 cm) was obtained on MS medium amended with optimum doses of IBA at 1.0 mg L^{-1} with AgNPs 1.0 mg L^{-1} (Fig. 2g). IBA combined with AgNPs treated cultures exhibited thick and long roots whereas, in the case of IBA and NAA alone supplemented culture showed slender and weak roots. Higher concentrations of auxins in the culture medium produce thin roots and intervention of callus formation was observed. Such shoots are not able to survive in the soil during the hardening process. It may be due to the presence of thin root and basal callus formations interfering with the connection between the shoot and root. Similarly, Sasidharan and Jayachitra (2017) reported that shoot tip explants gave 100% response of rooting, with about 10 roots per shoot in medium augmented with 1.5 mg L^{-1} IBA in *Enicostema axillare*. Roots are the first target tissues that come in direct contact with AgNPs in the culture medium and could be a reason for the uptake of nutrients from the medium. Similar results are documented on rice in vitro culture amended with AgNPs (Manickavasagam et al. 2019). Plantlets with well-grown roots were transferred and hardened in the greenhouse with a survival rate of 80%. Hardened plants showed morphological similarity with their parental plants and successfully survived under field conditions.

Assessment of genetic fidelity analysis was carried out through RAPD and SCoT markers after successful hardening (Supplementary Tables 5 and 6). Amplification of DNA was performed for micropropagated plants as well as the mother plant. For RAPD analysis 9 primers were selected for amplification, in that all the primers produced 35 homogenous and monomorphic bands in the range of 300–1000 bp (Fig. 3a), while 8 SCoT primers exhibited 21 monomorphic bands (Fig. 3b). No polymorphic bands were observed in all the regenerated plants. RAPD and SCoT markers revealed no genetic variability between the mother plant and micro propagated plants. Similarly, monomorphic banding pattern profiles were also documented in diverse plants such as *O. europaea* (Bradaï et al. 2019) S. *indicum* (Elayaraja et al. 2019), and N. *arbortristis* (Rath et al. 2020).

The present study describes a rapid and efficient methodology for the in vitro propagation of *H. enneaspermus* and the regeneration of multiple shoots from shoot

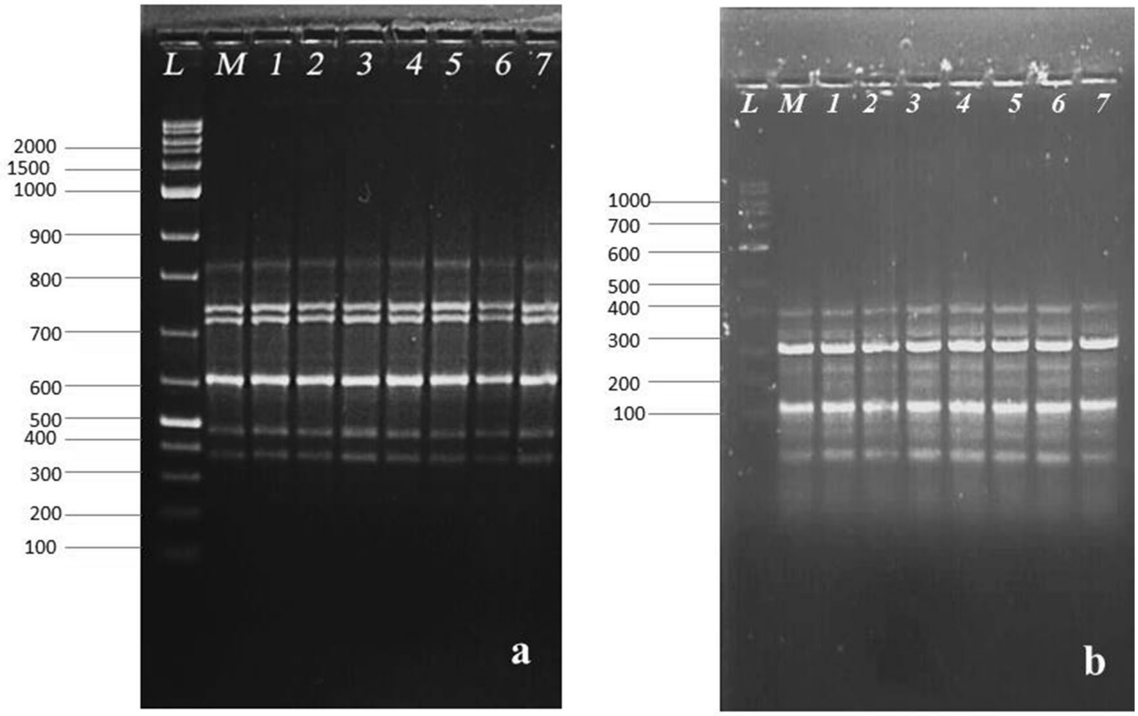

Fig. 3 Genetic fidelity analysis of in vitro regenerated plants and in vivo grown plant of *H. enneaspermus*. **a** RAPD primer OPA 01. Lane M-In vivo grown plant DNA, lanes 1–7 In vitro regenerated plants DNA, Lane L—1 kb plus ladder, **b** SCoT primer S4. Lane M—In vivo grown plant DNA, lanes 1–7 In vitro regenerated plants DNA, Lane L—1 kb plus ladder

tip explants. Improved shoot production was observed in the shoots exposed to AgNP-treated media. Multiple shoots (77.23 per explant) were produced with the highest percentage of response (90.66%) achieved on BAP and AgNPs amended MS medium. Thus, green synthesized AgNPs can be efficiently added to the plant tissue culture medium as an enhancement for improved micropropagation. Furthermore, we concluded that bio-fabricated AgNPs could be a pathway for practical implications of nanotechnology in biological sciences.

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Author contributions SS performed experiments, analyzed, compiled data and prepared manuscript. VV helped to carry out AgNPs synthesis. SK, CA and SPP contributed to results analysis. RS evaluated the data. MM supervised the findings of this work and evaluated the manuscript.

Declarations

Conflict of interest The authors declared that they have no conflicts of interest to this work.

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