RESEARCH ARTICLE

IN VITRO MICROPROPAGATION OF STACHYTARPHETA JAMAICENSIS L.VAHL., AN ETHNOMEDICINAL PLANT AND CONFIRMATION OF GENETIC FIDELITY OF THE PLANTLETS USING RAPD MARKERS.

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Abstract

In vitro shoots proliferated from shoot tip explants on MS medium supplemented with various concentrations of BAP, KN & TDZ and rooting of individual shoots occurred on MS medium supplemented with various concentration of IBA, IAA & NAA in Stachytarpheja jamaicensis L. Vahl. Maximum number of shoots & longest shoot length (cm) (1.96 ± 0.17) & (2.34 ± 0.16) respectively, were produced on MS + TDZ (0.5mg/L), after 4 weeks of culture. Maximum number of roots and longest root length (cm) (2.67 ± 0.88) & (3.33 ± 0.33) respectively, were produced on MS + IBA (0.5 mg/L), after 4 weeks of culture. The rooted plantlets (R₁) were acclimatized in greenhouse for 4 weeks and then transferred to field conditions with 69% survivability. RAPD primer OPE-20 banding pattern of R₁ plantlets was similar to that of mother plant.

Introduction:

Stachytarpheja jamaicensis L.Vahl (Verbenaceae), commonly known as Kukurdanti (Gervao), is native to Brazil, Cuba, Mexico and India. It is an annual and sometimes perennial herbaceous plant that grows 1.3 m tall and 1.6 m wide, before stems droop and touches the ground ¹. It has been in use as a traditional medicinal plant for many years; leaves are used to treat dysentery and intestinal worms ². In many countries, it is in use as herbal medicine for curing allergies, stomach problems, intestinal parasites, for stimulating digestion, suppressing cough, reducing fever, expelling worms, increasing perspiration, promoting menstruation, treating diarrhea and also has antimicrobial activity³. S. jamaicensis is also used as an antacid, analgesic, anti-inflammatory, diuretic, hypertensive, laxative, sedative, stomach tonic, spasmodenic, vulnerary and vermifuge⁴. Externally it is used to clean ulcers, cuts and wounds ⁵. In Malaysia, a decoction of the leaves is used as a draught for ulceration of the nose and as an anti-periodic medicine in malaria.

Several phytochemicals have been reported from S. jamaicensis such as alkaloids ⁶, ipolamide, hispidulin, beta-hydroxyipolamide, verbascoside⁷, steroids, triterpenes and irridoids⁸. Verbascoside has many potential applications such as, it is used as an antimicrobial compound ⁹ against many bacterial pathogens, including Staphylococcus aureus¹⁰. It is also in use as an anti-inflammatory compound ¹¹. Similarly, verbascoside is reported to have anti-
proliferative properties against human lymphocytic cancer cells. It is well known that verbascosides interacts with PARP-1 and p53 proteins and shows inhibitory activity on the protein kinase C enzyme.

In recent times, S. jamaicensis has been exploited commercially for its phytoconstituents and there is an urgent need to conserve this plant species for present and future use of its medicinal compounds.

Materials and Methods:-
Preparation of explants:-
Healthy shoot tips of S. jamaicensis were obtained from 6-8 months old well established plants growing in the research field of the Department of Botany, Kakatiya University, Warangal, India. The shoot tip includes the apical dome and 3-4 leaf primordia. The harvested shoot tip explants were washed under running tap water thoroughly and then rinsed with Tween-20 detergent solution for 10 minutes.

Preparation of MS Media:-
Full strength plant tissue culture media was prepared by taking 4.4 grams of readymade MS medium powder (Himedia) and 25 grams of sucrose in 500 ml of distilled water and the final volume was made up to 1000 ml. The pH of the media was adjusted to 5.8 using 0.1 N NaOH and the media was solidified with 0.8% agar before autoclaving.

Inoculation:-
The explants were surface sterilized with 0.1% HgCl₂ for 2-3 minutes under laminar air flow conditions and then washed with sterile distilled water. Shoot tip explants ranging from 0.5 to 1.0 cm in length were inoculated on to MS medium with different concentrations (0.5, 1.0, 1.5 or 2.0 mg/L) of Benzylaminopurine (BAP), (0.5, 1.0, 1.5 or 2.0 mg/L) of Kinetin(Kn) and (0.5, 1.0, 1.5 or 2.0 mg/L) of Thidiazuron (TDZ). All cultures were maintained in a culture room at 25±2°C with a relative humidity of 70 % and a 16 hours photoperiod at a photon flux density of 18-20 µE m⁻² s⁻¹ from cool white fluorescent tubes.

In vitro Rooting:-
Well developed shoots measuring 2.5 cm in length were cultured separately on MS medium with different concentrations (0.25, 0.50, 0.75 or 1.00 mg/L) of Indole-3- acetic acid (IAA), or Indole-3- butyric acid (IBA) or Naphthalene acetic acid (NAA).

Acclimatization and hardening:-
Rooted plantlets (R₁) were transferred to MS basal medium and grown under diffuse light in the culture room for 3 weeks. They were then transferred to plastic pots containing a mixture of sand, loamy soil and vermiculite in the ratio of 2:1:1(w: w) and grown in a green house for another 4 weeks. Hardened R₁ plantlets were transplanted in the departmental research field.

RAPD Analysis:-
Genomic DNA was extracted from 1g fresh leaf tissue of 03 randomly picked R₁ plantlets and a control (mother) plant using the CTAB method. DNA concentration and quality were evaluated at 260 and 280 nm. The A₂₆₀/₂₈₀ ratio ranged between 1.8 and 1.9. DNA integrity was confirmed by gel electrophoresis on 1% Agarose on TAE buffer (10M Tris HCl, 10M EDTA, pH 8.3). The RAPD primers (OPE 1-20) (Bioserve, Hyderabad) were used for PCR amplification of genomic DNA of R₁ plantlets and the control (mother) plant. PCR amplification was carried out in 20µl reaction volume containing 2.0 µl of 1.25 mM each of dNTPs, 1 µl of the primer, 1x Taq polymerase buffer, 0.5 U of Taq DNA polymerase (GeNei, India) and 2 µl (20ng) of genomic DNA.

DNA amplification was performed in a DNA Thermocycler (Biorad USA) which was used for initial DNA denaturation at 95°C for 5 min, 1 min annealing at 37°C and 2 min extension at 72°C, followed by one final extension at 72°C for 10 min. Amplified products were resolved by electrophoresis on 1.2% (w/v) Agarose (Sigma, USA) gel in Tris-Borate EDTA (TBE) buffer, stained with Ethidium bromide and photographs were taken by a gel documentation system (Biorad USA). The size of the amplification products was estimated using a 3 kbp ladder (Takara, China). All the reactions were repeated at least thrice to check for reproducibility.
**Statistical Analysis:**

All the experiments were repeated thrice with 12 replicates. The effect of different treatments was summarized as mean ± S.E and the data were subjected to statistical analysis using Duncan’s Multiple Range Test (DMRT) at 5% & 1% level significance.

**Results and Discussion:**

Percentage of shoot regeneration, mean number of shoots per culture and shoot length (cm) was scored in shoot tip explants cultured on MS media supplemented with either BA or Kn or TDZ at 0.5, 1.0, 1.5 & 2.0 mg/L each, in S. jamaicensis, after 4 weeks of culture (Fig-1-3). Highest percentage of shoot regeneration (88, 72, 60), maximum number of shoots/explant (1.96 ± 0.17, 1.72 ± 0.24 & 1.68 ± 0.04) with longest shoot length (cm) (2.34 ± 0.16, 1.66 ± 0.28 & 1.62 ± 0.19) was observed on MS + TDZ (0.5 mg/L), MS + Kn (1.0 mg/L) & MS + BA (1.5 mg/L) respectively.

Similar reports regarding the usage of BA, Kn and TDZ in shoot proliferation and multiple shoot induction from nodal and shoot tip explants are available in literature. Efficiency of BA in causing shoot regeneration from shoot tip explants of Vitex agnus-castus has been reported from nodal segments in Vitex trifolia. Sujatha et al. has induced the multiple shoots (4.55 ± 0.25 and 3.70 ± 0.25) in sponge gourd (Luffa cylindrica L.) by using 1.5 mg/L concentration of BAP from leaf and nodal explants respectively. Rohela et al. has induced multiple shoots (6.1 ± 0.64 and 6.7 ± 0.52) from nodal and shoot tip explants respectively in Rauwolfia tetraphylla L. by using 2.5 mg/L concentration of BAP.

TDZ was reported earlier as a potent cytokinin in inducing shoot proliferation in shoot tip and nodal explants of different plants and also TDZ is quite stable in in vitro culture conditions and it is resistant to cytokinin oxidases, hence it presently used by most plant tissue culturists across the world. Rohela et al. has used the MS+TDZ+BAP combination for the in vitro shoot regeneration from calli derived from leaf and stem explants of Rauwolfia tetraphylla L.

The proliferated shoots of S. jamaicensis were then tested for root induction by transferring the individual shoots on to different types of auxins (IBA, NAA & IAA) containing media (Fig-4-6). Among different auxins used, IBA has shown good response in root induction; more number of roots with maximum root length (cm) (2.67 ± 0.88) & (3.33± 0.33) was obtained on MS + IBA (0.5 mg/L) after 4 weeks of culture. Even though root initiation was also observed on NAA and IAA containing media, the results were not comparable to that of IBA.

In vitro plantlet formation from shoot tip explants of S. jamaicensis grown on MS medium supplemented with various concentration of BAP, Kn, TDZ, IBA is given in Fig 7 a-f. The rooted plantlets (R1) were hardened in pots containing 2:1:1 ratio of sand, loamy soil and vermiculite and then they were acclimatized initially in green house for 4 weeks and then transferred to field conditions with 69% survivability.

Genetic fidelity of plantlets was confirmed by using RAPD primers. Among the different primers used, RAPD primer OPE-20 has shown 08 bands in R1 plantlets within the range of 0.3 Kbp to 1.3 Kbp which was similar to that of control (mother) plant (Fig-8). RAPD primers were earlier reported for both DNA profiling and genetic fidelity studies in several plant species. Prasad et al. has reported DNA profiling of chilli peppers by using RAPD primers. Similarly, RAPD primers were used for analyzing genetic fidelity of Populus deltoides, Morus alba, Swertia chirayita.
**Fig. 1:** In vitro shoot proliferation from shoot tip explants of *S. jamaicensis* cultured on MS+ BAP (0.5-2.0 mg/L), after 4 weeks of culture

**Fig. 2:** In vitro shoot proliferation from shoot tip explants of *S. jamaicensis* cultured on MS+ Kn (0.5-2.0 mg/L), after 4 weeks of culture
Fig. 3: *In vitro* Shoot proliferation from shoot tip explants of *S. jamaicensis* cultured on MS+ TDZ (0.5-2.0 mg/L), after 4 weeks of culture.

Fig. 4: *In vitro* root induction in shoots of *S. jamaicensis* cultured on MS+ IBA (0.25-0.75 mg/L), after 4 weeks of culture.
Fig. 5: *In vitro* root induction in shoots of *S. jamaicensis* cultured on MS+ NAA (0.25-0.75 mg/L), after 4 weeks of culture

Fig. 6: *In vitro* root induction in shoots of *S. jamaicensis* cultured on MS+ IAA (0.25-0.75 mg/L), after 4 weeks of culture
Fig 7: *In vitro* shoot proliferation in shoot tip explants of *S. jamaicensis* grown on MS Medium supplemented with various concentration of BAP, Kn, TDZ, IBA after 4 weeks of culture

a) Shoot proliferation on MS+0.5mg/l and 1.5mg/l BA after one week culture

b) Shoot proliferation on MS+1.0mg/l and 1.5mg/l Kn after two weeks culture

c) Shoot proliferation on MS+0.5mg/l TDZ after two weeks of culture

d) Separated shoot cultured on MS+ IBA (0.5mg/l)

e) Root induction in shoots on MS+0.5mg/l IBA

f) Fully developed plantlet growing in pot after hardening process
Fig 8: RAPD profiling of R₁ plantlets and control (mother) plant of Stachytarpheta jamaicensis using OPE-20 primer:

Line M: Marker,
Line 1: Control (mother) plant of S. jamaicensis grown in research field,
Line 2-4: R₁ plantlets of S. jamaicensis grown in research field,

Conclusion:
In the present study an efficient protocol for in vitro proliferation of multiple shoots from shoot tip explants in S. jamaicensis was developed. RAPD marker (OPE-20) was used to assess the genetic fidelity was demonstrated in R₁ plantlets. So the present study can be applied for clonal propagation of medicinally important S. jamaicensis.

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Conflict of Interest:
Authors do not have any conflict of interest

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