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RESEARCH ARTICLE

GENETIC FIDELITY ANALYSIS OF ENCAPSULATED MICROSHOOTS OF *SARCOSTEMMA BREVISTIGMA* USING RAPD MARKERS.

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Abstract

In vitro grown microshoots of *Sarcostemma brevistigma* were used as explant for encapsulation. Encapsulated microshoots were kept at 25°C, and the success rate of regrowth was found to be approximately 50% following 1 months of storage. Encapsulated microshoots showed 37.66% formation of multiple shoots on plant growth regulator free Murashige and Skoog (MS) medium. Healthy root formation was observed in all microshoots following two weeks of transfer on half-strength MS medium containing 0.5 µM α-naphthalene acetic acid and 0.5 µM Indole acetic acid. These plants were subsequently transferred to pots containing a mixture of soil, sand and farm yard manure (2:2:1, v/v), and then shifted in the greenhouse. In the green house and the overall survival was found to be 75% after 2 months. The genetic fidelity analysis of *S. brevistigma* plants developed from encapsulated microshoots was done using random amplified polymorphic DNA (RAPD) marker. For molecular analysis 25 decamer primers were used to check genetic fidelity of plants selected from all three batches was carried out using. Only 5 primers produced scorable amplified products and a total of 38 bands were observed; out of these 10.52% bands were polymorphic. Cluster analysis of the RAPD profile revealed an average similarity coefficient of 0.95 confirming genetic stability of plants derived from encapsulated microshoots following 1 months of storage at 25°C.

Abbreviations:-

MS	Murashige and Skoog medium
m amsl	meter above mean sea level
NAA	α-naphthalene acetic acid
IAA	Indole acetic acid
UPGMA	Unweighted pair-group method with arithmetic mean
RAPD	Random Amplified Polymorphic DNA
RET	Rare, endangered or threatent

Introduction:-

Sarcostemma brevistigma Wight & Arnott (Asclepiadaceae) grows throughout India and other tropical regions of the world. According to folklore, this plant extract is used for the treatment of asthma. A fraction of this plant extract has been reported to have anti-allergic and anti-inflammatory activities (Saraf and Patwardhan, 1988). It is found to be active as anti rheumatic, anti-allergy, antiemetic and bronchodilator (Kirtikar and Basu, 1993). Dried stem is an

emetic employed in leprosy patients. Roots have been used in snake bite and rabies. The plant contains malic acid, succinic acid, reducing sugar, sucrose, traces of tannin and alkaloid a phyto sterol, α and β- amyrine, lupeol and lupeol acetate and β-sistosterol. Phytochemical studies reveal the presence of bergenin, brevine, brevinine, sarcogenin, sarcobiose and flavonoids (Oberai et al., 1985, Khare et al., 1980, Khare et al., 1987).

Due to great medicinal impact the plants species are collected from the wild to meet the industrial demand. Hence, these plants are decreasing rapidly in natural habitat. Using available information based on field survey, it is assumed that the other causes of its degradation are largely overexploitation, low regeneration in the natural habitats and clearing of forests for developmental activities. Therefore, conservation of this important medicinal plant to biotechnological approaches is urgently required.

Establishment of ex situ conservation facilities for plant germplasm in the form of field gene banks, seed gene banks, in vitro collections and cryogenically preserved tissues is a common practice (Rao, 2004; Borner, 2006). Encapsulation using alginate medium provides a feasible approach for in vitro germplasm conservation (Are et al., 2000).

The potential advantages of "synthetic seeds" are, its genetic stability and ease of handling and transportation, along with increased efficiency of in vitro propagation through space, time, labour and overall cost (Nyende et al., 2003). Although "synthetic seeds" have been widely used for micropropagation and conservation of various medicinal plant species (Anand and Bansal, 2002; Manjkhola et al., 2005; Singh et al., 2006 a, b; Narula et al., 2007; Ray and Bhattacharya, 2008; Lata et al., 2009), the genetic stability analysis of "synthetic seed" has been started recently. The ever increasing use of synthetic seeds for germplasm conservation and propagation necessitates assessment of genetic stability of propagules, particularly following storage (Dehmer, 2005).

In recent years, the use of DNA marker technology has become common a practice. Among different techniques available, e.g., RFLP, AFLP, ISSR and RAPD, the latter has been widely used to study clonal integrity, detect genetic and somaclonal variations (Jokipii et al., 2004; Borner, 2006; Mandal et al., 2007; Agnihotri et al., 2009). RAPD markers are often used because of their easy handling, cost effectiveness, simple genotyping, easy availability and being non-radioactive, etc.

In this study, conservation of *S. brevistigma* germplasm, using microshoots, called "synthetic seed" technology has been undertaken to ensure steady supply of quality plants. This is first report of synthetic seed development in *S. brevistigma*. This study will generate an impact on sustainable collection of quality raw material for medicinal use, for collection of elite germplasm, rehabitation of species by regenerated plantlets, establishment of nurseries and their plantation to control over-exploitation in natural habitats and maintain its posterity for future.

Material and Methods:-

Plant Material:-

Plant samples of *S. brevistigma* Wight and Arnott. (Common name: Somvalli) were collected from Kyonti region of Rewa District, MP, India. Samples were brought to the laboratory and used to develop in vitro cultures. In vitro proliferating shoot cultures were used as a further source of explants. Shoot tips and nodal segments from these were excised (3-5 mm), and used as explants for encapsulation.

Encapsulation:-

Individual components used for gelling and encapsulation, namely half-strength Murashige and Skoog (MS; 1962) medium, 3% sodium alginate (Sigma, St. Louis, MO, USA), 1.5% (w/v) solution of sucrose and 3% (w/v) calcium chloride solution ($CaCl_2$) were autoclaved at 120 °C for 20 min under 15 lbs pressure. Shoot tips and nodal segments excised from in vitro proliferating shoots of S. brevistigma were suspended in the sterile sodium alginate gelling mixture, and then dispensed drop-wise into the $CaCl_2$ solution under continuous gentle shaking. The resultant beads further referred as encapsulated microshoots; average dia 0.8 mm were allowed to remain in the $CaCl_2$ solution for 30 min to complete the ion-exchange reaction, and these encapsulated microshoots were then washed thoroughly with sterilized distilled water. The encapsulated microshoots were stored in a moist-environment in a flask for 1 months at 25± 2 °C; moist conditions were maintained by spraying encapsulated shoots with sterile distilled water (in the form of mist) at 15 days interval. After 1 month of storage, the encapsulated microshoots were placed on plant growth regulator (PGR)-free semi-solid half strength MS basal medium (0.4%, w/v, phytagel) for regrowth at 25± 2 °C under 16 h photoperiod (cool fluorescent tubes, Philips 40 W; 42 and 60 µ mol/m^2/s irradiance inside and

outside the culture flasks, respectively). Data on regrowth of synthetic seeds were recorded after 2, 3 and 4 weeks of culture. Developing shoots without roots were further transferred to half-strength MS medium supplemented with 0.5 µM α-naphthalene acetic acid and 0.5 µM Indole acetic acid for rhizogenesis

Healthy plants from each batch were transferred to earthen pots containing a mixture of soil, sand and farm yard manure (2:2:1, v/v) and kept in the greenhouse with irradiance reduced to 50% of ambient. In vitro raised *S. brevistigma* plants, used as explants source, were also established in the greenhouse and served as "Mother plant" (control).

RAPD analysis:-
After 60 days of growth in the green house, three randomly-selected plants (from three baches) derived from encapsulated microshoots, along with control plant were used to RAPD analysis. DNA isolation was performed according to Doyle and Doyle (1987) with modifications.

A total of 25 random decamer oligonucleotides (Operon Technologies Inc., Alameda, California, USA) were used as single primers for the amplification of RAPD fragments. RAPD analysis was performed using the method of Williams et al. (1990). Polymerase Chain Reactions (PCRs) were carried out in a final volume of 25 µl containing 20 ng template DNA, 200 µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 and 0.5 U Taq DNA polymerase (Bangalore Genei, India). Amplification was achieved in a Thermocycler (Biometra; Germany) programmed for a preliminary 5 min denaturation step at 94ºC, followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 37ºC for 1 min and extension at 72ºC for 1 min, and finally at 72ºC for 10 min.

The amplified products were separated by electrophoresis on 1.5% agarose gels run in 1X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha ImagerTM IS-2200, San Leandro, CA, USA). PCRs were repeated at least twice to establish reproducibility of results.

Data Analysis:-
Each polymorphic band was considered as a binary character and was scored 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Only intensely stained, unambiguous, and reproducible bands were scored for analysis. Similarity index was estimated using the Dice coefficient of similarity (Nei and Li, 1979). Cluster analyses were carried out on similarity estimates using the unweighted pair-group method with arithmetic-mean (UPGMA) using Gene Profiler 1-D Phylogenetic analysis & Data basing Software.

Results and Discussion:-

After 1 months of storage at 25± 2 °C in the light, and under moist conditions, the encapsulated microshoots of S. *brevistigma* were capable of regrowth within 2 weeks of culture (Fig. 1). The frequency of regrowth was 49.66% after 4 weeks of culture on PGR-free MS medium. This observation substantiates an earlier report involving *Picrorhiza kurrooa* where encapsulated microshoots demonstrated approximately 90% germination after 3 months of storage in sterile water (Mishra et al., 2011). Amongst the responding encapsulated microshoots of *S. brevistigma*, 37.66% exhibited formation of multiple shoots at the onset of regrowth; while single shoot formation per encapsulated microshoot was recorded in 28.33% cases (Fig. 2). Emergence of a single or multiple shoots from one encapsulated explant indicates the presence of one or more shoot primordia per explant, which has also been reported earlier (Shrivastava et al., 2009). Further, 27.33% encapsulated microshoots exhibited simultaneous formation of shoots and roots.

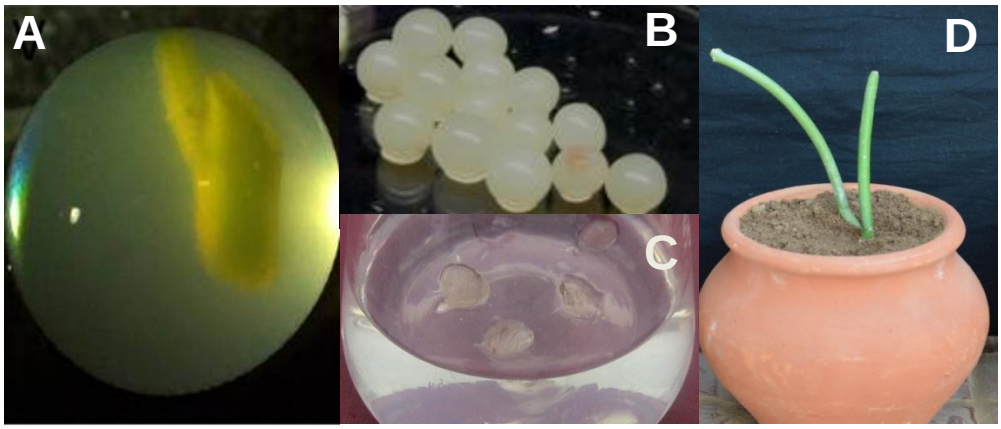

Fig. 1:- Encapsulated microshoots of *S.brevistigma* and their response after storage at 25±2°C.
a & b. Calcium alginate beads formed by encapsulation of explants **c**. Alginate beads placed on PGR-free MS medium for regrowth; **d**. Healthy plants derived from regrowth of encapsulated microshoots after 6 weeks of transfer to the green house.

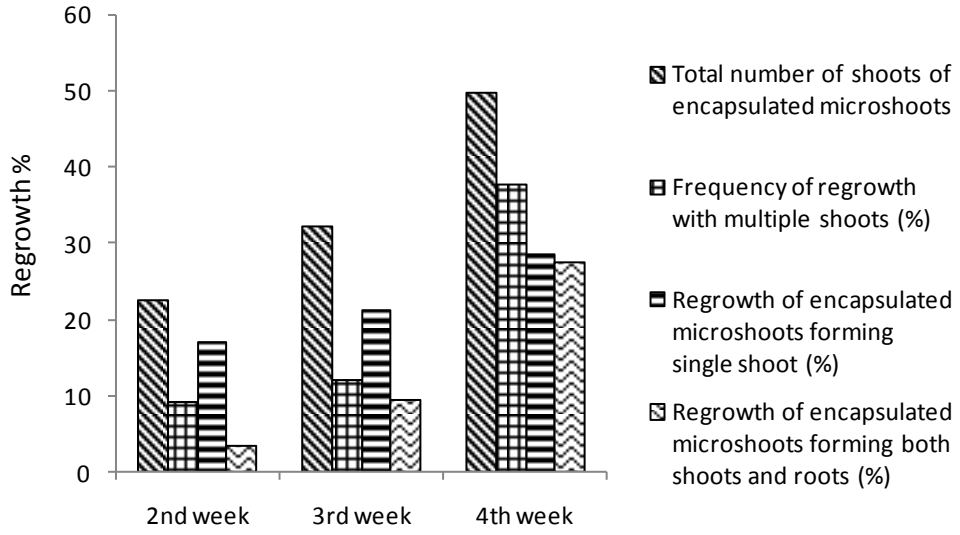

Fig. 2:- Frequency of regrowth of encapsulated microshoots of *S. brevistigma*.

The conservation of germplasm through encapsulation has been reported in a number of medicinal plants, such as *Ocimum species* (Mandal et al., 2000), *Adhatoda vasica* (Anand and Bansal, 2002), *Withnia somnifera* (Singh et al., 2006b), *Rauvolfia serpentina* (Ray and Bhattacharya, 2008), *Cannabis sativa* (Lata et al., 2009), etc. The development of whole plants through encapsulated microshoots is being reported for the first time. This study also reports successful storage of encapsulated microshoots of *S. brevistigma* at 25± 2°C, in contrast to many of the above cited reports where storage has been carried out at 4 °C. Therefore, the present study is important for developing a cost effective conservation strategy, without involving maintenance at low temperature (0°C or 4°C).

In the present study half strength MS medium was found suitable for regrowth of encapsulated microshoots. Addition of different PGR combinations, reduction in the concentrations of sucrose as well as that of other nutrients to half appears to be an essential prerequisite in the present study in order to optimize the efficiency of shoot regeneration.

A total 25 random primers were tested for the assessment of genetic fidelity of the plants derived from encapsulated microshoots. Out of these, only 5 primes produced clear and scorable amplified products. Of the 38 amplification products only 10.52% bands were polymorphic, while the rest were monomorphic (Fig. 3). The number of bands for each primer varied from 5 (OPA-3) to 10 (OPC-19, Table 1) and ranged between 0.2 to 2.9 kb (Fig. 3). Amongst the 5 amplified primers, 3 primers produced monomorphic bands and only 2 primers produced polymorphic patterns (Table 1). Lower frequency of polymorphic bands has also been reported in *Abies cephalonica* (Aronen et al. 1999), *Melia azedarach* (Scocchi et al., 2004) and *Cineraria maritime* (Shrivastava et al., 2009).

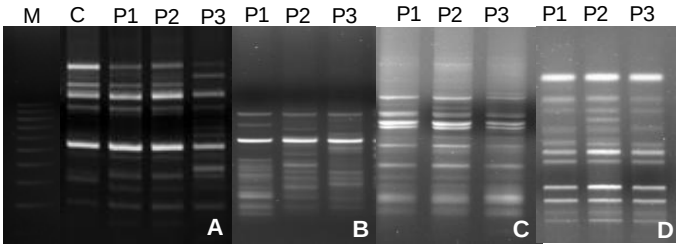

Fig. 3:- RAPD amplification profiles with primers OPA- 2 and OPC- 7, 18 & 19; M molecular weight markers; C: Mother or control plant; P1-P3, plants derived from the regrowth of encapsulated microshoots of *S. brevistigm*, randomly taken from the three batches of regrown plants.

Table 1:- Sequence of 5 primers that resulted in clear and amplified products used in RAPD profiling of regenerated plants of *S. bravistigma*

Primer	Primer sequence	Total No. of amplified products	No. of polymorphic bands	% polymorphic bands
OPA-2	5' TGCCGAGCTG 3'	8	2	25
OPA-3	5'AGTCAGCCAC 3'	5	0	0
OPC-7	5'GTCCCGACGA 3'	8	2	25
OPC-18	5'TGAGTGGGTG 3'	7	0	0
OPC-19	5'GTTGCCAGCC 3'	10	0	0
Total		38	4	10.52

A similarity matrix calculated from the RAPD analyses was subjected to cluster analysis using the UPGMA. Average value of similarity matrix was 0.95. Thus the results clearly indicate that plants derived from regrowth of encapsulated microshoots after storage resembled the parent clone at the genetic level.

Findings of this study highlight the importance of encapsulation technique which can be efficiently utilized for propagation of medicinally important plants. The results of this study on *S. brevistigma* further support the feasibility of this cost effective and simple germplasm conservation approach for storage and regeneration of true-to-type plants.

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