



Original Research Article

Novel low cost culture media “KFA and KFA plus” For micropropagation of *Mentha* sps.

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A B S T R A C T

Keywords

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A low cost novel plant tissue culture medium “KFA and KFA plus” (Patented) has been developed using “Flyash” (FA) as the main source of inorganic constituent in the medium. It has been used in various combinations with and without nitrogen source, and plant growth regulators. To study the efficacy of this media with 5%, 7%, and 10% of flyash was used for micropropagation of *Mentha arvensis* and *M. spicata*. Growth of the plants in KFA and KFA+ medium was compared with the most widely used Murashige and Skoog’s (MS) medium (control). After standardization all media were supplemented with PGRs IAA (0.50mg/l) + BAP (1.00mg/l). Comparisons were done on the basis of percent bud break, percent shooting and average shoots/explants. Efficient results were obtained, showing healthy, normal growth of *Mentha arvensis*, *Mentha spicata* grown in KFA and KFA plus cultures were found to be similar to growth in control medium. The percent shooting was similar in both media (MS control) and KFA, KFA plus. 83.3% shooting and an average of 1.27 shoots/explants were observed in fly ash grown culture. The efficacy on the basis of percent shooting and shoots/explants were found to be better in 7% and 10% in case of *M. arvensis* whereas 5% in case of *M. spicata*. An average of 4555 shoots was obtained in *M. arvensis* and 2596 shoots in *M. spicata* after three subsequent subculturing. Hundred percent rooting was achieved in media containing IBA (1.0mg/l) + BAP (0.5mg/l). 100% survivability was achieved when these rooted plants were transplanted into pots. Genetic homogeneity of the in vitro generated plants was ascertained by DNA profiling. Fly ash in the KFA and KFA plus culture medium resolves our aim of low cost plant production and also the disposal problem of the thermal power plant waste (FA) up to a large extent.

Introduction

Plants with its innumerable benefits have been exploited by mankind for their own wellbeing. Furthermore rapid increase in population has led to an increase in food

and energy demand many folds. In comparison to the developed countries food situation in most of the developing countries is precarious. To meet the food

demands, production has to be increased and the increased production has to be obtained from a declining cultivable land area.

Plant tissue culture (PTC) holds the key to accelerated food production and medicines by continuous production and supply of plantlets of desired variety/genotype and thereby reducing the use of land area. Micropropagation technology via plant tissue culture provides better approach to raise clones of plants in a large number in which rapid proliferation is achieved and has been widely applied for the production of a large number of economically important plants including valuable medicinal plants, trees, staple food crops, horticultural plants.

However commercialization of such technologies has been hampered by high production cost (Kozai et al., 1997). The cost of components of tissue culture medium has been also a concern for most commercial laboratories (Babbar and Jain, 2006). The composition of the culture media used for proliferation has a tremendous influence on production costs. Therefore low cost tissue culture technology is the need of the day. Low cost tissue culture technology is the adoption of practices and use of equipments to reduce the unit cost of plant production without compromising the quality of the plants. Agar and sucrose are the chief constituents which play a significant role in cost of production. Attempts have been made for the use of cheaper alternatives of agar like starch, gelrite and isabgol (Pierik, 1991; Gebre and Sathyanarayana, 2001; Kodym and Zapata, 2001; Nagamori and Kobayashi, 2001; Jain and Babbar, 2006, 2011; Raghu et al., 2007). The low cost alternatives of sucrose (Bonaobra et al, 1994; Agrawal et

al., 2009) such as household sugar and other sugar sources can be used to reduce the cost of medium. Table sugar as an alternative low cost medium component for *in vitro* micro-propagation of potato; *Solanum tuberosum* L. was used by Demo et al., (2008).

Reduction in the cost of basal salts can also contribute to the reduction in the unit price of tissue cultured plants. In an attempt to achieve this and to reduce the cost of tissue cultured plants, Biswas and Biswas (2009) developed and patented (Patent No. 1113/del/2009 dated 01/06/2009) a novel plant tissue culture medium "KFA and KFA plus" using "Flyash" as the main source of inorganic constituent in the medium. In general, 95-98% of FA consists oxides of Si, Al, Ca and about 0.5-3.5% of Na, P, and S (Grey and Lin, 1972.). Jala (2006) revealed useful ameliorant nature of FA, improves properties of problem soils. It is a source of readily available plant macronutrients like K, P, Ca, Mg, S. and micronutrients like Fe, Zn, Cu, Mo, B, Mn. In the present study it was used in various combinations with and without nitrogen source, and plant growth regulators. To study the efficacy of this media varying percentage of flyash was used for micropropagation of *Mentha arvensis* and *M. spicata*. *Mentha* species are considered highly popular industrial crops as they are a source of essential oils enriched in certain monoterpenes, widely used in food, flavor, cosmetic and pharmaceutical industries. Plantlets derived from tissue culture are known to exhibit somaclonal variation (Larkin and Scowcroft, 1981), which is often heritable. Random Amplified Polymorphic DNA (RAPD) and microsatellite polymorphism analysis can be used to detect variation at DNA level (Zhang et al., 2001; Yadav et al., 2012).

Genetic homogeneity studies of the regenerated plants were carried out using PCR based Microsatellite polymorphism.

Materials and Methods

Source of Explant material

Plants of *Mentha spicata* and *M.arvensis* were collected from Sarangpur, Botanical Garden, Chandigarh.

Subject studied

Fly ash used in study was collected from the dumps of thermal power plant, grey in colour and having pH-8.0.

Establishment of culture

Aseptic cultures of both the plants were established from the apical and nodal explants with one node and a pair of axillary buds. The explants were surface sterilized with 70% ethanol for 30 seconds followed by 0.1% (w/v) mercuric chloride for 5 minutes and rinsing with sterile distilled water thrice and inoculated in KFA, KFA plus which had Flyash as the main source of inorganic constituent and also in MS (Murashige and Skoog 1962) medium used as control. KFA and KFA plus media were tested with various concentration of Flyash (10%, 7% and 5% w/v). KFA plus was supplemented with nitrogen source comprising of Glycine 2mg/l, Nicotinic acid 0.5 mg/l, Pyridoxine-HCl 0.5mg/l, Thyamine-HCl 0.1mg/l while KFA was devoid of nitrogen source. All the three media were supplemented with 3% sucrose (w/v) as carbon source, 0.8% agar (w/v) and plant growth regulators (PGRs) in the combinations/concentrations of IAA (0.50mg/l) and BAP (1.00mg/l), IAA (1.00mg/l) and BAP (0.50mg/l), IBA

(0.50mg/l) and BAP (1.00mg/l), NAA (0.50mg/l) and BAP (1.00mg/l). The pH of all media was set to 5.7. All cultures were set in ten replicates for each combination and the experiments were repeated thrice. The cultures were maintained at $25\pm 2^{\circ}\text{C}$, 1500 lux, photoperiod of 16hrs light/8hrs dark regime and 70% humidity. The cultures were routinely observed for any contamination. Different growth parameters like bud break with respect to number of days after inoculation, % shoot growth, average shoots/explants, % rooting and morphological characters of regenerated plantlets. Mean (\bar{X}), Standard Deviation (σ) and Standard Error were calculated for induction frequencies of the explants and % shooting. All statistical analysis was performed by using SPSS software program.

DNA isolation and Microsatellite polymorphism analysis

DNA isolation of the mother plants and the regenerated plants were carried out using the modified CTAB method technique without liquid nitrogen (Biswas and Biswas, 2011). After checking the purity of DNA they were subjected to PCR amplification for microsatellite profiling using the primers: PMS1 (GTG)₅, PMS2 (GAC)₅ and PMS3 (GACA)₄ separately. Each reaction was set up for 50 μl mixture consisted of the following reagents:

Template DNA 2.0 μl (0.1 μg), 10X Taq buffer with 15mM magnesium chloride 5.0 μl (1X), Magnesium chloride (25mM) - 1 μl (0.5mM), dNTPs (2mM) - 5.0 μl (0.2mM), Primer (PMS1,PMS2,PMS3) - 1.0 μl (20 pmoles), *Taq polymerase* - 0.25 μl (5U/ μl), autoclaved distilled water - 37.5 μl subjected to an amplification cycle of initial denaturation at 94°C for 5 min, 39 cycles of (denaturation at 94°C for 15

seconds, annealing at 55°C, 45°C, 47°C for PMS1, PMS2, PMS3 respectively for 45 seconds, extension at 72°C for 1:30 min) and final extension at 72°C for 7 min in the Eppendorf Mastercycler Personal Thermal Cycler.

After the completion of the cycles the sample was tested for amplification by electrophoresing on 1.2% gel using 5µl amplified PCR product and 4µl loading dye at a voltage of about 66 V for 60 to 90 minutes. 100 bp ladder marker was used for size determination. Bands were observed under Gel Documentation System.

Results and Discussion

Initial cultures of *Mentha arvensis* and *Mentha spicata* were successfully standardized and established in full strength MS medium and on low cost medium using fly ash of varying concentration supplemented with growth regulators. Out of the four combination of growth regulators used, the best result was observed in IAA (0.50mg/l) and BAP (1.00mg/l). All the data presented are the results of this combination.

Effect of fly ash on bud break

The percent bud break was calculated and the best results were observed in KFA plus (7% FA) medium for *M. arvensis* and KFA plus (5% FA) for *M. spicata* as compared to control MS medium. In *M. arvensis* 86.66% of bud break was noticed in about 8 days in KFA plus (7%FA) medium and 86% in KFA (7%FA) in 16 days as compared to 80% in MS in 7 days. In *M. spicata* 76.6% of bud break was noticed in about 14 days in KFA plus (5%FA) and 73% of bud break in 23 days KFA (5%FA) as compared to 78.8% in MS in 15 days.

Effect of fly ash on percent shooting

Plants were subcultured in all the three media supplemented with PGRs IAA (0.50mg/l) + BAP (1.00mg/l) and mean % shooting, SD± SE were calculated. Data shows KFA plus (7%, 10% FA) and KFA (7% FA) gave 83.33% as compared to 80% in MS medium for *M. arvensis*. Mean shoot /explants was found to be 1.27 in KFA plus (7%FA) and 1.23 in both KFA (7%FA) and MS (Fig.1, Table.1, Plate.A). Percentage shooting in *M. spicata* was highest i.e 75% and 74% in KFA plus (5%, 7% respectively) followed by MS (72%) and KFA (70%). Mean shoot /explants was found to be 1.27 and 1.23 in KFA plus (5%, 7% FA respectively) and 1.23 in MS followed by 1.03 in KFA (5% FA) (Fig:2, Table.1, Plate. B). An average of 4555 shoots was obtained in *M arvensis* and 2596 shoots in *M spicata* after three subsequent subculturing (Fig.3).

Stock solutions of macro-nutrients and micro-nutrients poses significant share in production cost of micropropagated plants. As the main objective of the study is low cost plant production in tissue culture, therefore fly ash was used as an alternative inorganic constituent in culture media in present work. This was the first time when FA has been used in tissue culture and no previous work on tissue culture using FA has been reported till now.

Interestingly in all the results the percent bud break, percent shooting and mean shoot/explants was found to be either more or same in both MS (control) and KFA plus (7% FA) medium for *M.arvensis* and MS and KFA plus (5% FA) for *M..spicata* (Table 1, Figs 1, 2). In KFA alone which was without nitrogen source but with PGRs, 73% to 83.3% shooting was

observed in *M. arvensis* and comparatively low (63 to 70%) in *M. spicata*. The variable response in the two plants in KFA may be attributed to the genotype response to the availability of nitrogen in the medium.

Multiple shooting was observed at the basal portions of the plant, after 3 subsequent subculturing an average of total 7151 shoots were obtained from basal, intermodal and nodal portions in both species of *Mentha*. (4555 shoots were obtained in *M arvensis* and 2596 shoots in *M spicata*).

It was very noticeable fact that with the decreasing concentration of Fly ash better response was obtained in *Mentha* species. Purple pigmentation around the leaves was reduced and leaves turned greener. This may be attributed to the fact that Fly ash consists of excessive macronutrients and micronutrients along with trace elements which were directly available to the plant triggering metabolites destined to produce secondary metabolites like menthol and other isoterpenoids derivatives which caused purple pigmentation around leaves.

One of the problems occurred during standardization of cultures in MS medium turned brown after bud break or did not develop into proper shoot. To overcome this, an anti browning agent 1% Activated Charcoal (AC) was added to MS medium to prevent oxidation by menthol in *Mentha arvensis* and carvone in *Mentha spicata* which might have caused browning of explants. Addition of activated charcoal also added cost to the medium and thus raised the cost of plants. But plant grown on FA did not show browning (minimising use of AC) reducing the cost of medium

and they were greener, even after subculturing. This data is in agreement with the previous review stating tomato plant grown on ash soil mixture showed luxuriant growth with bigger and green leaves. Plant growth, yield, (flowering, fruiting, fruit weight/plant), carotenoids and chlorophyll were mostly enhanced in the treatment with 40-80% fly ash, being optimal at 50-60%, from 60-70% onwards, the measured parameters tended to reduce. At 100% fly ash, yield was considerably reduced (Khan and Khan, 1996).

Rooting and hardening

Fifty percent of the shoots were transferred in rooting medium and 50% of the shoots were kept under propagation in shooting medium. Shoots of about 2-3cm length were transferred in all three media containing IBA (1.0mg/l) + BAP (0.5mg/l) and extensive fibrous roots were obtained from each plantlet within 15 to 25 days (Fig.4, Plate:C). The plants survived well in the rooting medium. Rooting in *Mentha arvensis* was also observed in most of the plantlets growing in the shooting medium with IAA (0.5mg/l) +BAP (1.0mg/l), but very fragile roots developed, hence they were transferred to the rooting medium. All shoots cultured in this medium, showed rooting. Therefore 100% rooting was observed.

Hardening was carried out in the special mixture soil (vermiculite:soil 1:1) under artificial conditions. Plants were given high to low humidity and low to high light intensity from sun. On hardening 100% plantlet survived (Plate:D).

Plantlets obtained from all different types of media were found similar to each other.

Table.1 Showing % shooting, mean shoot/explants, SD±SE, of *Mentha arvensis* and *Mentha spicata* on KFA and KFA plus media with varied concentration of Flyash and MS (control) medium supplemented with PGR combination of IAA (0.50mg/l) + BAP (1.00mg/l)].

Media type	<i>M. arvensis</i>				<i>M. spicata</i>			
	Mean shoot/explant	SD±SE	% shooting	SD±SE	Mean shoot/explant	SD±SE	% shooting	SD±SE
MS	1.23	0.23 ±0.129	80%	10.0 ±5.77	1.23	0.225 ±0.032	72%	1.73 ±0.99
KFA (10%FA)	0.93	0.05 ±0.032	73.30%	5.77 ±3.33	0.93	0.057 ±0.032	63.3%	5.77 ±3.33
KFA (7%FA)	1.03	0.06 ±0.032	83.30%	5.77 ±3.33	0.96	0.02 ±0.0115	64%	5.29 ±3.054
KFA (5%FA)	0.93	0.152 ±0.087	73.30%	11.54 ±6.66	1.03	0.057 ±0.032	70%	10.0 ±5.77
KFAplus(10%FA)	1.23	0.06 ±0.032	83.30%	5.77 ±3.33	0.76	0.115 ±0.066	71.8%	9.50 ±5.48
KFAplus (7%FA)	1.27	0.15 ±0.087	83.30%	5.77 ±3.33	1.23	0.225 ±0.032	74%	5.29 ±3.05
KFAplus (5%FA)	1.10	0.10 ±0.057	73.30%	5.77 ±3.33	1.27	0.152 ±0.087	75%	1.0 ±0.58

Fig.1 Showing percentage shooting and mean shoots/explants of *M. arvensis* in all the media type supplemented with PGR combination of IAA (0.50mg/l) + BAP (1.00mg/l)

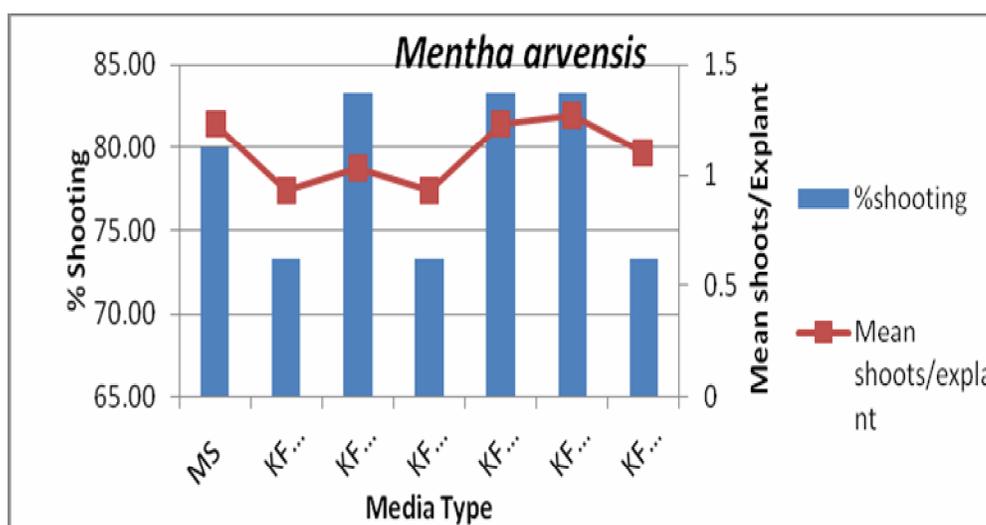


Fig.2 Showing percentage shooting and mean shoots/explants of *M.spicata* in all the media type supplemented with PGR combination of IAA (0.50mg/l) + BAP (1.00mg/l).

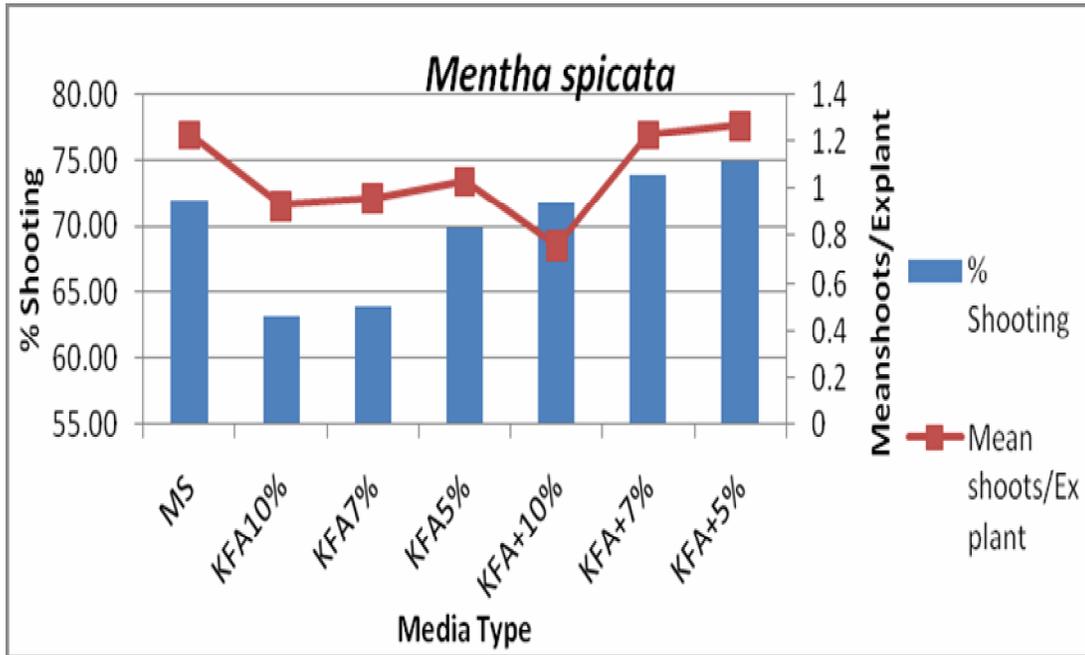


Fig.3 Showing total no. of shoots obtained after three subsequent subculturing

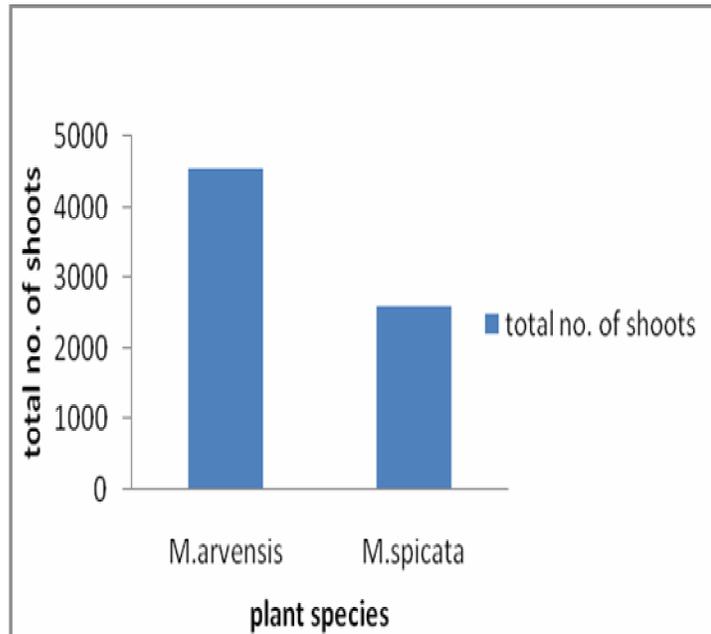
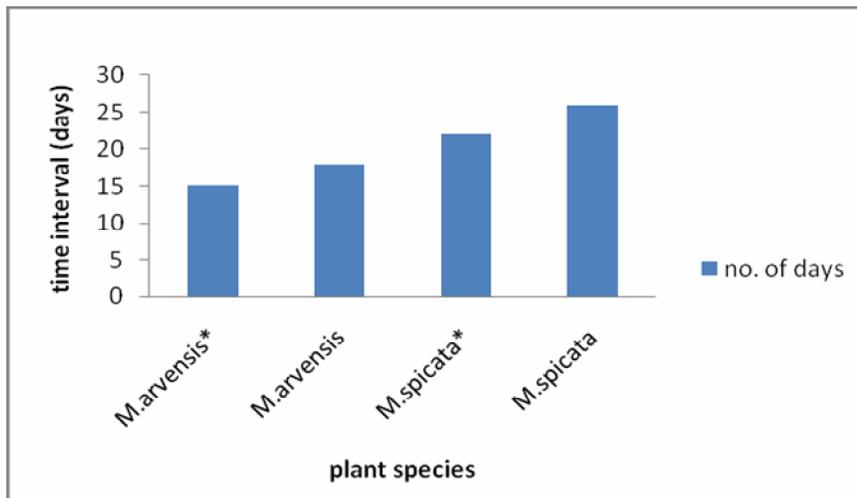


Fig.4 Showing rooting with time interval (days) observed in *Mentha* species



*denote PGRs concentration IAA(0.50mg/l) + BAP(1.0mg/l). Without denote PGRs concentration IBA (1.0mg/l) + BAP (0.5mg/l)

Plate A: Showing plantlets of *Mentha arvensis* after subculturing on KFA plus (7% FA) medium with IAA (0.5mg/l) + BAP (1.0mg/l).

Plate B: Showing plantlets of *Mentha spicata* after subculturing on KFA plus (5% FA) medium with IAA (0.5mg/l) + BAP (1.0mg/l).



Plate C: Showing profuse root growth of *M. arvensis* in KFA plus medium supplemented with IBA (1.0 mg/l) + BAP (0.5mg/l).

Plate D: Healthy plants of *M. arvensis* in pot after transfer to soil.

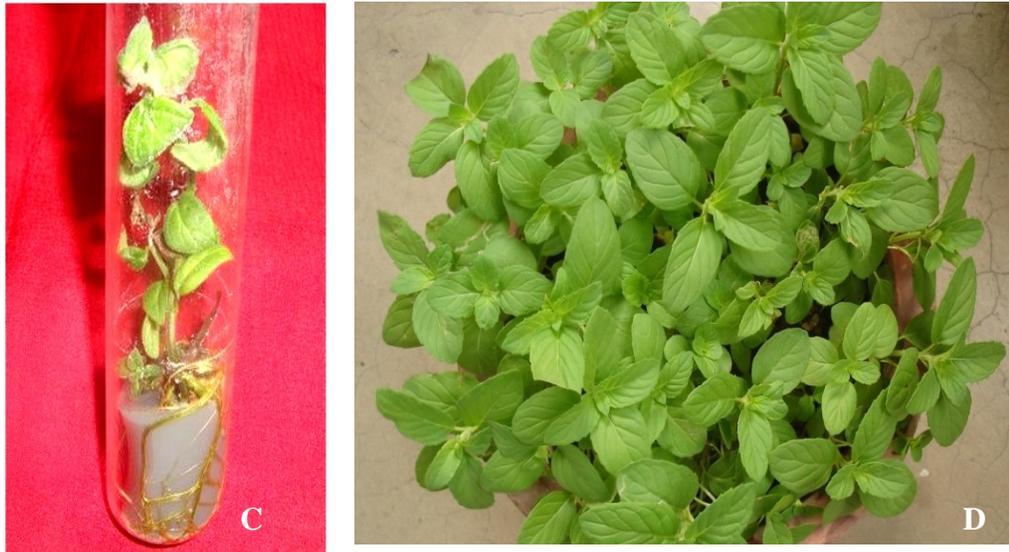
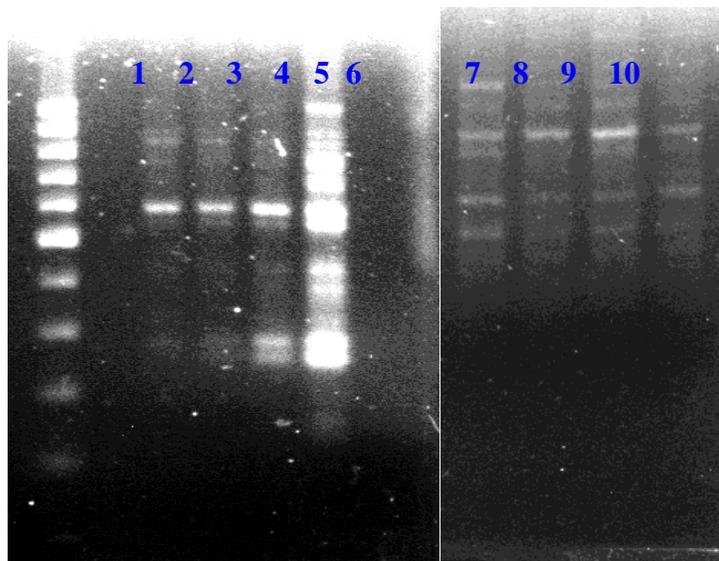


Fig.5 Microsatellite with primer PMS1 (GTG)₅ of *M. spicata* (Lanes 2-6) and PMS2 (GAC)₅ of *M. arvensis* (Lanes 7-9). Lane 1- 100bp ladder,



Certain morphological differences were seen when the plants growing in vitro were compared with those growing ex vitro like increase in the inter nodal length, adventitious shoots arose from basal calli in case of some plants, extensive fibrous and fragile roots were developed which were greater than 5-7 cm in length. However, all the plantlets showed normal growth after they were transplanted to soil.

Genetic homogeneity studies by Microsatellite fingerprinting

Four to six amplified bands ranging between 150-800 bp with PMS1, between 300-800 bp with PMS2 and between 100-600 bp with PMS3, were observed in *Mentha spicata* samples from all media samples. Six amplified bands ranging between 300bp to 1000bp with PMS1, six bands between 150bp- more than 1000bp with PMS2 and two bands between 500bp to 1000bp with PMS3 were observed in all media types of *M. arvensis*. Same banding pattern was observed in all media types of both the *Mentha* species (Fig.5).

Of several molecular markers used for such assessment, RAPD and microsatellite are the simplest, cheapest and appears to be a useful tool for the analysis of genetic fidelity of in vitro propagated plants. RAPD has been widely used to assess the genetic fidelity of in vitro regenerated plants by several workers (Gururaj and Uddagiri 2012; Mallaya et al, 2013) while microsatellite based assessment is rare. Microsatellite being more robust, its application for genetic fidelity assessment is preferable. In this study same banding pattern with microsatellite primers in all the plants shows that genetic homogeneity was maintained in the regenerated plants. This noteworthy data emphasizes the advantage of using Fly ash over

constituted MS medium (M1 as control), in terms of economy, shoot multiplication rate, availability of plant irrespective of seasonal variation and fulfills the goal of micropropagation. The use of Fly ash also deals with the problem of its disposal, leading to bioremediation.

As plants propagated on MS (control) medium and FA medium showed similar morphological patterns of growth therefore use of FA medium is a better substitute to lower down the cost of the tissue culture medium as FA is available free of cost from thermal dumps. Fly ash as the culture medium resolves our aim of low cost plant production and also the disposal problem of the thermal power plant (TPP) waste (FA). The use of FA in PTC established during this study using *M. arvensis* and *M. spicata* from axillary buds of nodal portions in culture can be channelized for in vitro cultivation of other commercially important plant species.

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