Micropropagation and attempts to develop RAPD molecular markers for salt tolerant cultivars of pearl millet

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Abstract - Differences were observed among four salt tolerant cultivars of pearl millet to in vitro culture media. Among the four cultivars, Cv IP 6098 is the most salt sensitive had maximum number of multiple shoots (15)/ seed, and the more salt tolerant cvs IP 6106 and IP 22269 developed 3-6 shoots/seed while cv IP 19612 the seeds remained single shooted in MS+3.0mg/L media up to 7 months. The multiple shoots 20% of cv IP 6098, flowered in 5 months with the reduction in hormone concentration (MS+1.0 mg/L BAP) whereas, the other three cultivars did not flower under the tested conditions.

The present study was carried to detect genetic differences among the four cultivars of pearl millet at molecular level through RAPD PCR markers. RAPD analysis was carried with 12 primers, each 10-mer, only 7 primers were amplified showed polymorphism for the four cultivars. Primer OPM 16 revealed a single unique band of ≈485bp in the genotype IP 22269 and three polymorphic bands in the genotype IP 6106. The results were repeatable, and further studies in this direction may enable to link the single band in the genotype IP 22269 to a morphological trait or physiological condition like salt tolerance.

***Index Terms*— Pearl millet, cultivars, *in vitro* flowering and Molecular marker.**

I. INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] is an important food and fodder crop grown in India, Pakistan and Africa. especially in desert regions. It is the sole cereal in hot and dry regions of India and Africa and that forms the staple food of such regions [1]. In pearl millet, there are large number of cultivars that can adapt to high temperatures, drought and also salinity. The plant is an open pollinated crop, it is difficult to selectively propagate or maintain a desired genotype that is resistant to salinity or high temperatures. Reproducible micropropagation techniques will be handy to propagate desired genotypes "true to type" condition. Among different micropropagation techniques, "Multiple shoot induction" is a preferred technique to other available clonal propagation techniques, because it is quicker than somatic embryogenesis and chances of somaclonal variation are a few. Multiple shoot induction with *in vitro* flowering confirms the normal physiology of the micropropagated clones.

In pearl millet a vast diversity exists at morphology and genotypic level [2,3]; development of DNA molecular markers will help us to understand the genetic diversity among the various cultivars and also link a marker to morphological/physiological/biochemical character. The first and significant contribution on molecular analysis of pearl millet came in 1994, when 181 RPLF markers were reported [3]. Subsequently many molecular markers have been generated and also mapped in pearl millet for disease resistance eg. downy mildew, [4] and drought tolerance [5,6]. However, the limitation with the molecular markers is, they are not always repeatable, less polymorphic and with restricted PCR compatibility [1]. Therefore, development of additional molecular markers with reproducibility would be a valuable addition to pearl millet research. In the present study 12 decamer, random primers are screened against the four orphan cultivars of pearl millet. We report that OPM 16 primer was polymorphic, revealed 3 bands in cv IP 6106 and a single unique band of ≈485 bps in cv IP 22269.

II. MATERIALS AND METHODS

Mature seeds from self-pollinated ear heads of four cultivars IP 19612, IP 6106, IP 22269 and IP 6098 were obtained from International Centre for Biosaline Agriculture, Dubai, UAE. To screen for salt tolerant seeds, for each cultivar 60 seeds were germinated in Petri dishes containing water (control) and/or 50mM NaCl solutions. The seeds were incubated at room temperature at 25°C under 12hr light and 12hr dark photoperiod.

One week old control and salt tolerant seedlings were surface sterilized with 0.01 % mercuric chloride for 15 mins and were washed with sterile water for 3-5 times. The surface sterilized seedlings were cultured in MS basal medium [7] supplemented with 3.0 mg/L BAP, 3% sucrose and 0.8% agar (Hi media, Mumbai, India) media. The multiple shoots were subcultured once after every 4-5 weeks. The multiple shoots were separated into individual shoots and were subcultured in MS+1.0 mg/L BAP for elongation of the shoots and for rooting. The cultures were maintained at 24±2°C with a photoperiod of 16/8 hr light/dark.

DNA extraction:

Genomic DNA was extracted from the leaf tissue of one month old field grown plants of cultivars IP 19612, IP 6106, IP 22269 and IP 6098, using Accuvis bio DNA isolation kit (Norgen, Canada) following the manufacturer's instructions. The extracted DNA samples were electrophoresed on 1% agarose gel at 100 V, the gels were then viewed and photographed under UV illumination.

RAPD PCR conditions:

A set of 12 random decamer primers that revealed polymorphism for different accessions of pearl millet [8] were used for PCR amplification; were purchased from

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(Macrogen, Korea). A 20 µl PCR reaction was performed containing 1µl (25-40 ng) of extracted genomic DNA, 10 µl 2X PCR master mix from Accuvis bio, 1µl (10µM) of each primer and 8 µl of sterile distilled water using a thermal cycler (Veriti PCR Machine, Applied Bio systems, USA). The PCR amplification conditions were 94ºC for 15 sec, 35ºC for 30 sec, and 72ºC for 60 sec followed by 45 cycles with a final extension at 72ºC for 10 min. The PCR products were electrophoresed on 1.8% agarose gel at 100 V, the gels were viewed and photographed under UV illumination.

Table 1: Random primers and their sequence used for RAPD-PCR analysis

S.No	Primer	Sequence(5'-3')
1	OPL-11	ACGATGAGCC
2	OPAL-20	AGGAGTCGGA
3	OPAL-08	GTCGCCCTCA
4	OPAL-15	GGGGACACC
5	OPM -16	GTAACCAGCC
6	OPN-16	AAGCGACCTG
7	OPF-10	GGAAGCTTGG
8	OPF- 01	ACGGATCCTG
9	OPE-04	GTGACATGCC
10	OPE-09	CTTCACCCGA
11	OPE-18	GGACTGCAGA
12	OPAG-13	GGCTTGGCGA

III. RESULTS & DISCUSSION

The seeds of the four cultivars incubated in water germinated in one day with 100 % germination in every cultivar. There was delay in germination of seeds, incubated in 50 mM salt solution by 2-3 days among all four cultivars. The seed germination in cv IP 6098 is 87%, while it is 94% - 96% in the other three cultivars. Among the four cultivars, IP6098 is the most salt sensitive cultivar, reported earlier [9].

A. Response of four cultivars to MS+3.0 mg/L BAP:

Sixty per cent (36/60) of one week old salt stressed seedlings of cv IP6098 developed each 5-6 multiple shoots by 6 weeks of culture. The shoots were short, bright green and there was no root formation for the multiple shoots. The multiple shoots did not elongate or develop roots if were retained in the same medium. The multiple shoots after about 2 to 2 ½ months were subcultured in MS+1.0 mg/L BAP medium for elongation of the dwarf shoots. The subcultured shoots elongated, developed roots and shoots up to 15/seed in MS+1.0 mg/L BAP by 3 months from the initial culture. The response of control seedlings was quicker to the culture media with similar per cent (6/10) of response and number of shoots (15-18)/seed.

The response of salt stressed seeds of cvs IP 6106 and IP 22269 were similar to that of cv IP 6098 except less number of shoots/seed developed in the culture media. In cv IP 6106, 40% of cultured seedlings formed 4-6 shoots/seed in 1.5-2 months; the shoots were longer, than those of cv IP 6098 and light green in color with no root formation. On the other hand, in cv IP 22269 each seed developed into 2-3 shoots which were much stunted in growth with the outer leaves turning necrotic. The seedlings of IP 19612 remained single shoots, did not develop any multiple shoots even after 5 months of culture in MS+3.0 mg/L BAP medium. The response of respective control seedlings to culture media was similar to that of the stressed seedlings, except that is faster. The control seeds of cv IP 19612, only 1 (1/10) formed two shoots/seed.

The results indicate that the hormone requirements for multiple shoot induction of salt stressed seedlings of the four cultivars of pearl millet are different and are genotype dependent. The genotype 6098 responded the best to 3.0 mg/L BAP producing up to 15 shoots/seed in 3 months' time (Fig.1), while cv IP 19612 could not be induced to form multiple shoots with this hormone level indicating either higher levels of BAP or other cytokinins necessary.

B. *In vitro* flowering of cv IP 6098:

The multiple shoots of cvs IP 6098 and IP 6106 were further subcultured in MS+1.0 mg/L BAP media for elongation of shoots and for rooting. After 4 months from the initial culture, the multiple shoots that are separated into single shoots, 20 % (7/36) of them bolt an inflorescence. If there were two shoots that could not be separated into individual ones during subculture, both of them flowered (Fig. 2). The inflorescence was about 2 inches long and protogynous with bifid stigmas emerging earlier than the anthers. The spikelets were white with glumes, ovaries and versatile anthers. *In vitro* flowering could not be induced in cv IP 6106 shoots and also in cv IP 22269 under the present protocol albeit the shoots were sub- cultured for 7 months in the media. These results indicate that hormone requirements for flower induction in cultures are different among the four cultivars of pearl millet. *In vitro* flowering was earlier reported in pearl millet from immature embryos [10] and shoot apices [11]. A decrease in the concentration of BAP from 3.0 mg/L to 1.0 mg/L triggered flowering in the cv. IP 6098, while it failed to do so in the cv IP 6106. The external hormone requirements for different genotypes of pearl millet are different [12].

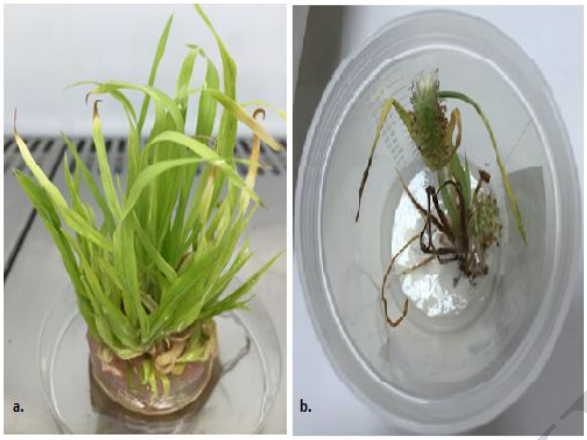
In vitro flowering was earlier reported in pearl millet cv Vg 272, in which flowering was induced in 2 months. The differences between the previous report and the present study are 1. In Vg 272 the explants were immature embryos, 2 BAP+NAA together induced flowering and 3. Multiple shoots en masse flowered while in the present study the explants are mature seeds which were induced to flower with BAP in the media, in about 5-6 months. It is

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well documented in literature that immature embryos and leaves respond to cultures faster than the mature explants. It can be speculated that the amount of florigen was adequate to trigger flowering when all the multiple shoots were together. Whereas in single shoots it would take more time to accumulate the required level of florigen. Overall, *in vitro* flowering in pearl millet appears to be more vulnerable to hormones as it could be triggered in multiple shoots induced from shoot apices [11], mature and immature embryos [10] than it is in other cereal crops.

Figures 1 &2: Multiple shoot induction and *in vitro* flowering of salt tolerant cv IP 6098 of pearl millet.

Fig.1: ≈15shoots/seed after 3 months in MS+ BAP media.
Fig.2: *In vitro* inflorescences in MS+1.0 mg/L BAP medium after ≈ 5 months of culture.



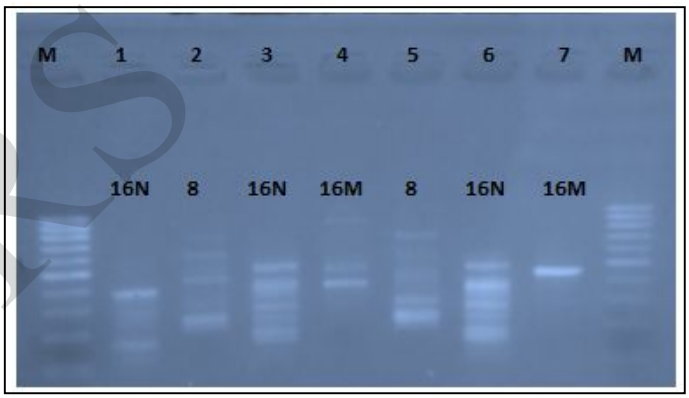
C.PCR - RAPD Analysis

The RAPD profile generated for each primer was analyzed using a DNA marker (100 bp DNA Ladder, Accuvis bio) and the experiment was performed in triplicates for each DNA sample, for different batches of leaf material to check the reproducibility of the generated RAPD profile. The results showed polymorphic and unique bands among the four cultivars. Only reproducible bands were considered for the analysis. The sizes of amplified fragments ranged from 150bp to 1000bp. From a total of 12 primers, 7 primers had 21 polymorphic bands, while 5 primers were not amplified among the four cultivars. The amplified primers were 4-7 among the cultivars (Table II). The results are interesting in that, primer OPN16 has similar bands in the cvs IP 6106 and IP 22269. OPM 16 displayed 3 polymorphic bands in the cv IP 6106 and a single band of ≈ 485bp with IP 22269.
RAPD primers are reported to reveal genetic polymorphism in other cultivars of pearl millet [8], wheat [13], Sorghum [14] and also barley [15]. The work is at a very primitive stage and continuation of the work is mandatory to understand the genetic distance and similarity among the four cultivars of pearl millet. The future work may lead us to relate the unique band to a morphological or genetic or biochemical trait in the cv IP 22269.

Table –II: Amplified RAPD primers for four cultivars of pearl millet.

Sl.No	Cultivar	No of bands	Primer Name
1	IP 19612	4	OPE4, OPL11, OPAL15, OPAG13, OPN16
2	IP 6106	7	OPE4, OPL11, OPAL15, OPAG13, OPN16,OPAL8, OPM 16
3	IP 6098	5	OPE4, OPL11, OPAL15, OPAG13,OPAL8
4	IP 22269	6	OPE4, OPL11, OPAL15, OPAG13, OPN16, OPM 16

Figure II: RAPD banding profile for four cultivars with different primers. **Lane 1- 2:** IP 19612, **Lanes 3-4:** IP 6106, **Lane 5:** IP 6098 and **Lanes 6 & 7:** IP 22269.



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