Induction of Androgenesis in Pearl Millet

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Abstract
Breeding efforts in Pearl millet (Pennisetum glaucum (L.) R. Br), one of the most widely cultivated drought- and high-temperature tolerant C4 cereals, are aimed at maximum exploitation of hybrid vigor for both grain and forage yields. Until now, very limited work has been carried out on in vitro production of haploids in pearl millet; while it is being employed as the pollinator which will be further eliminated, resulting in haploids of the recipient species, e.g. wheat, oat. Anther culture experiments were carried out with seven genotypes 841-P3, 843-22B, ICMB 93333, ICMB 89111, XL-51, 4201 and 86-M34 tested on 12 different culture media. Androgenic embryos were induced in the frequency of 13.7, 9.51 and 7.58 % from 841-P3, ICMB 93333 and XL-51 cultivars. Inclusion of 4% maltose as additional carbon source resulted in higher number of multicellular microspores among the responsive genotypes. These experiments form a promising basis to further develop double haploid protocol for pearl millet breeding in the arid and semi-arid regions.

Keywords Anther Culture, Pearl Millet, Double Haploids, Pro-Embryo

1. Introduction
Pearl millet (Pennisetum glaucum (L.) R. Br) is one of the most widely cultivated drought- and high-temperature tolerant C4 cereals. If pulse is the poor man's meat, millet is the poor man's bread. It is equal or even superior to rice and maize in protein and oil content (http://www.cgiar.org/our-research/crop-factsheets/millet/). Breeding efforts in pearl millet are aimed at maximum exploitation of hybrid vigor for both grain and forage yields. Hybrid parents are bred mostly by recurrent selection which was based on the natural out-breeding behavior of the crop; or by pedigree breeding from populations that have mostly been derived from single crosses. This approach continues to be widely used and quite successful, although the obvious effect is narrowing of the genetic base of hybrid cultivars. Cultivars developed from OPVs (open pollinated varieties) and hybrids are highly heterogenous and heterozygous, open to significant genetic changes. Such cultivars developed from single progenies are marred by inbreeding depression and loss of vigor (Rai et al. 1999). Though NILs and RILs hold great importance in breeding and mapping for traits, their limitations are the long time and/or high cost required for development, and these populations only detect the additive component but do not provide information on dominance relationships for any QTL (Semagn et al. 2010). On the other hand, DH are quicker to generate than NILs and RILs, and they can also be used to generate NILs (Mithila and Hall 2003).

Pearl millet is being exploited in breeding programs to develop DH but merely as the pollinator which will be further eliminated, resulting in haploids of the recipient species, e.g. wheat, oat etc. (Marcinska et al. 2012). Until now, very limited work is carried out on in vitro production of haploids in pearl millet (Dang Ha and Pernes, 1982; Nitsch, 1982; Choi et al. 1989). Ha and Pernes (1982) were the first to report successful in vitro androgenic haploids in pearl millet lines Tifl23D2B and a F1 hybrid. In the same year, Nitsch (1982) reported haploid regeneration from in vitro cultures of anthers. Vasil and Haydu (1981) carried out studies on somatic embryogenesis of a related species Pennisetum purpureum and obtained diploids from in vitro culture of anthers. However, the entire regenerated plants were from the cells derived from the anther wall. Mallikarjuna (1987) examined the suitability of different culture media for pearl millet anther culture and obtained multicellular microspores on MS media, but regeneration of whole plants was not achieved. Further efforts are demonstrated by one report (Choi et al. 1989), which focused on two genotypes for refining the technique of androgenesis and regeneration. Considering the fact that most successful protocols for DH through anther and/or microspore culture belong to cereal species i.e. rice, barley, wheat and maize; only sporadic work has been done in pearl millet. Expression studies towards understanding of androgenesis for potential markers have also been carried out in cereals making them model species for DH production. Maize is the third closest relative of pearl millet followed by rice, the first and second being foxtail millet and sorghum (Rajaram et al. 2013). The limited amount of sequence
information in pearl millet has limited progress in gene
discovery and characterization, global transcript profiling,
probe design for development of gene arrays, and generation
of molecular markers and their application in crop
improvement programs. With this rich background in cereals,
and the growing interest in pearl millet breeding in the arid
and semi-arid regions, it makes a very interesting study to
explore technique of anther culture for the induction of
androgenesis.

2. Materials and Methods

i. Donor plants: Experiments were carried out with
seven genotypes 841-P3, 843-22B, ICMB 93333,
ICMB 89111, XL-51, 4201 and 86-M34 obtained
from Genetic Resources Unit, ICRISAT
(International Crops Research Institute for the
Semi-Arid Tropics), Patancheru, India. Plants were
grown in the greenhouse maintained at 25/20 °C
(day/night) under natural light conditions with the
intensity varying from 450–600 µmol m⁻² s⁻¹. A
3:2:1 ratio of red soil, sand and vermin-compost
mixture was autoclaved and filled in 30 cm pots with
six plants per pot and a minimum of three pots per
variety were maintained. In order to have a
continuous supply of florets, fresh batch of sowing
was done once the spikes from primary branches
have been harvested. All the pots were labeled
appropriately. From January to April, the cultivars
were grown in the field with temperatures ranging
from 22±2 °C (min) to 32±2 °C (max).

ii. Sample, Pre-treatment of buds and sterilization:
Spikes/inflorescence of pearl millet still enclosed in
the flag leaf, were collected between 8-9 am both
from the greenhouse and the field. Whole spikes
were cold pre-treated at 4 °C for either 2 or 7 days.
The spikes with and without cold treatment, after
removing the flag leaf were surface sterilized using
0.1% Mercuric chloride with 2-3 drops of Tween 20
for 15 min, washed thrice with sterile distilled water
to remove all traces of the surfactant. Care should be
taken to dispose of mercuric chloride appropriately
as mixing with ground water causes mercury
contamination. The microspore developmental stage
was confirmed by squashing one anther (out of three
present in each spikelet) removed from the spikelet,
on a microscopic slide in a drop of 2% acetocarmine
and observed for microspore developmental stage.
Only the portion of the spike containing uni-nucleate
microspores was used for culture initiation and rest
of the spike was discarded.

Culture media, anther culture and observations:
Direct culture of anthers and microspores was
observed to non-responsive (Mallikarjuna, personal
communication) and culture of florets was reported
to be most effective (Ha and Pernes, 1982). Hence,
sessile florets from sterilized spikes were carefully
and aseptically removed with forceps and placed
vertically on the solidified agar media. A total of 12
different media were tested with Ch1 media (also
used in chickpea anther culture) as the base and
varied combination of growth regulators (Table. 1).
After the initial induction period, florets with
emerged anthers were transferred to growth medium,
while some were retained on the original media.
Periodic observations were carried by 2%
acetocarmine squashes and observation under light
microscope.
Table 1. Media Used for Anther Culture Experiments in Pearl Millet

| Components (mg/lit) | CH1 |
|---------------------|-----|
| NH₄NO₃             | --  |
| KNO₃               | 2500.0 |
| KH₂PO₄             | 170.0 |
| NaH₂PO₄.H₂O        | --  |
| CaCl₂.2H₂O         | 600.0 |
| Ca(NO₃)₂.4H₂O      | --  |
| MgSO₄.7H₂O         | 370.0 |
| FeSO₄.7H₂O         | 27.8 |
| Na₂EDTA            | 37.3 |
| H₃BO₃              | 6.2  |
| KI                  | 0.83 |
| MnSO₄.H₂O          | 16.9 |
| ZnSO₄.7H₂O         | 1.4  |
| CuSO₄.5H₂O         | 0.025 |
| NaMoO₄.2H₂O        | 0.25 |
| CoCl₂.6H₂O         | 0.025 |
| Thiamine HCL       | --   |
| Pyridoxine HCL     | --   |
| Nicotinic acid     | --   |
| Myo-inositol       | --   |
| L-Glutamine        | --   |
| Glycine            | 5.0  |
| Cholecalciferol (vitamin D) | -- |
| Cynacobalamin (vitamin B₁₂) | -- |
| Calcium pantothenate | -- |
| Riboflavin         | --   |
| Glutathione        | --   |
| Biotin             | --   |
| Casein hydrolysate | 200.0 |
| Coconut water (ml) | --   |
| Picloram           | 0.265 |
| 2-ip               | --   |
| IAA                | --   |
| Kinetin            | --   |
| 2,4-D              | 0.530 |
| BAP                | 0.09 |
| Sucrose            | 17,000 |
| Maltose            | --   |
| pH                 | 5.8  |
| Agar (%)           | --   |

| Media (mg/lit) | Auxin | Concentration (mg/lit) | Cytokinin | Concentration (mg/lit) |
|----------------|-------|------------------------|-----------|------------------------|
| CHNB           | NAA   | 2                      | BAP       | 1                      |
| CHNZ           | NAA   | 2                      | Zeatin    | 1                      |
| CHNT           | NAA   | 2                      | Thidiazuron | 1                    |
| CHNK           | NAA   | 2                      | Kinetin   | 1                      |
| CHN2           | NAA   | 2                      | 2ip       | 1                      |
| CH2B           | 2,4-D | 2                      | BAP       | 1                      |
| CH2Z           | 2,4-D | 2                      | Zeatin    | 1                      |
| CH2T           | 2,4-D | 2                      | Thidiazuron | 1                    |
| CH2K           | 2,4-D | 2                      | Kinetin   | 1                      |
| CH22           | 2,4-D | 2                      | 2ip       | 1                      |
3. Results

i. Effect of donor plant: Androgenic ability greatly depended on genotype of the explant with varied response among the seven genotypes tested. Maximum average response with respect to anthers with viable microspores after 7 days in culture was observed in ICMB 93333 (92.02%) followed by XL-51 (85.33 %) and 841-P3 (84.75%) and the response was least in the genotype 4201 (45.12 %). However, in terms of number of multi-nucleate to multi-cellular microspores per anther, maximum response was observed in 841-P3 (13.7 %) followed by ICMB 93333 (9.51 %) and XL-51 (7.8 %) with the least being 4201 (0.21 %) (Fig. 1).

ii. Effect of cold pre-treatment: The panicles of pearl millet that were subjected to cold treatment for 2 and 7 days were non-responsive without any divisions, and turned pale within a week of culture. Anthers emerged out from the florets within 5-8 days in culture when no cold pre-treatment was applied to the i.e. the controls. Also, direct culture of control and pre-treated anthers on semi solid and liquid media with different combinations of plant growth regulators failed to respond. No divisions were observed and anthers turned brown in color after 7 days with pale and empty microspores after 15 days in culture.

iii. Effect of culture media/growth regulators: The culture medium composition varied with respect to growth regulators, CH medium being the basal medium. Initial experiments were carried out CH + 1.7 % sucrose, with different combinations of auxins and cytokinins. Each of these media contained one auxin i.e. either 2-4D or NAA in combination with one cytokinin i.e. Zn, Kn, TDZ, 2-ip and BAP. These ten media combinations when used for induction of androgenesis, elicited varied responses under light and dark conditions. Out of these ten combinations, emergence of anthers from florets and higher percentage of viable microspores were observed in NAA+Kn, 2-4D+Zn and 2-4D+2-ip under light condition. While six of the ten media i.e. 2-4D+Zn, 2-4D+Kn, 2-4D+2-ip, NAA+BAP, NAA+TDZ, NAA+2-ip and NAA+Kn responded in dark condition (Fig. 2). However, after 15 days in culture, multi-nucleated microspores were observed only in 2-4D+2-ip and NAA+2-ip under dark phase. Increase of sucrose concentration to 8 % from earlier 1.7 %, enhanced the viability of microspores and also resulted in higher number of dividing microspores under dark incubation conditions in NAA+2-ip and 2-4D+2-ip media. Another medium N6+2-4D+BAP also yielded multi-nucleated and multicellular microspores from three of the most responsive genotypes i.e. 841-P3, ICMB 93333 and XL-51. Continuous cultures on these three media effectively induced multi-cellular microspores by 15 days in culture. Transfer of responsive florets on to a growth medium consisting of CH+8% sucrose+4% maltose+2-4D+BAP, (after 15 days in induction media) yielded pro-embryoids and globular embryos after 10 days in culture.
iv. Effect of carbon source and concentration: The availability of carbon at different stages of growth had a significant effect on the rates of induction and production of multi-cellular microspores. The sources of carbon were sucrose and maltose in the experiments conducted. Sucrose was used in two concentrations 1.7 % and 8 % for both induction and further divisions. The anthers from the florets cultured on the 8% sucrose media emerged out early when compared to 1.7% sucrose media for all the genotypes tested. Number of viable microspores and induction rates were higher when cultured in media with 8 % sucrose (Fig. 3). After 15 days in culture on 1.7 % sucrose medium, most microspores appeared to be pale, light stained and shiveled with very few 4-8 nucleated microspores. In contrast, most microspores were slightly enlarged, round and bright stained with many multi-nucleated microspores with dividing cell walls in high (8%) sucrose concentration media. At this stage, transfer of florets to medium containing 8 % sucrose and 4 % maltose resulted in many large multi-cellular microspores containing more than 20 cells per diving microspore. 10 days in this medium post induction in 8 % sucrose media for 15 days, resulted in pro-embryoid like structures in the genotype 841-P3 and many multi-nucleate and multi-cellular microspores in other genotypes ICMB 93333, XL-51 and 843-22B (Fig. 4). However, direct culture of florets on high carbon containing 8 % sucrose + 4 % maltose media did not generate multi-cellular microspores although the viability of microspores was comparable to sucrose (sole source of carbon) containing medium.
4. Discussion

Among the seven pearl millet varieties tested, 841-P3 was the best performer resulting in pro-embryoids while 4201 was least responsive. Unlike chickpea, where there is a stark difference between the most and least responsive genotypes, most of the genotypes of pearl millet responded with considerable percentage. Sucrose is the most common source of carbohydrates used in plant tissue culture in a concentration of 2-4%. Some crops such as Brassica species and cereals prefer higher concentrations of 12-13% and 6% respectively. Substitution of sucrose by maltose is known to have genotype-independent plant regeneration and direct embryogenesis in wheat (Bhaojwani and Dantu 2013). In all the genotypes tested in pearl millet experiments, the average response and number of multicellular microspores induced were higher in 8% sucrose media compared to 1.7% sucrose. Pro-embryoids were induced when cultured in CH media with 8% sucrose and 4% maltose. Addition of maltose in half the concentration of sucrose significantly enhanced the androgenic response. Addition of brassinosteroids and arabinogalactans to the media which support both induction and growth could potentially reduce the use of multiple media at different developmental stages. These experiments form a promising basis to further develop double haploid protocol in pearl millet breeding which could influence breeding efforts in sorghum, another important cereal of the arid and semi-arid regions.

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