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Original Research Article

Standardize Protocol for Callus Induction and Plant Regeneration in Barnyard Millet using Different Combination of Plant Growth Regulators

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A B S T R A C T

The greatest progress towards the development of nutrient medium for plant cells grown in culture took place in 1960 and 1970 (Gamborg and Miller. 1968). The basic nutritional requirements of cultured plant cells are very similar to those utilized by plants. However, the nutritional composition varies according to the cells, tissue, organs, and protoplasts and also with respect to particular plant species. A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic supplements which include organic nitrogen, acids and complex substances (Gautheret. 1942).. The plant hormones really act alone and for most processes at least these that are observed at the organ level. Many of these regulators have interacted in order to produce the final effect. The classical plant hormones (Auxins, Cytokinins, Gibberellins, Abscisic acid, Ethylene) and growth regulatory substances with similar biology effect (Murashige T and Skoog F.1962) at the same time, novel structurally related compounds are constantly being synthesized. More recently discovered natural growth substances that have Phytohormone like regulatory roles (Polyamines, Oligosaccharins, Salicylates and Jasmonates). Many of these growth active substances have not yet been examined in relation to growth and organized development *in vitro*.

Introduction

Barnyard millet (*Echinochloa frumentacea* and 2n=27) is one of the oldest domesticated millets in the semi-arid tropics of Asia and Africa. It is a staple cereal in areas where climatic and edaphic conditions are unsuitable for rice cultivation (Yabuno, 1987). There are about 20 species in the genus *Echinochloa*; two are cultivated namely *E. frumentacea* (Indian barnyard millet) and *E. esculenta* (Japanese barnyard millet). *E. frumentacea* originated in India and possibly also in Africa. It is an annual cultivated form in India, Central African Republic, Tanzania and Malawi (Dogget, 1989 *E. frumentacea* is grown for grain, fodder and beer, although not as extensively as in the past..In India, barnyard millet is grown from Himalayan region in the north to Deccan plateau in the south. It is cultivated over an area of 1.95 lakh ha,

mainly in the states of Uttarakhand, Madhya Pradesh, Karnataka, Uttar Pradesh and North east region of India (Seetharam, 2011). Though in the recent years, the crop has gained renewed interest as a health-food due to its rich nutritional profile and high dietary fiber content. Nutrient composition of barnyard millet is protein 10.52 g, fat 3.56 g, fiber 10.1 g, energy 398 kcal, carbohydrate 65.5 g, minerals 4.4 mg, calcium 11 mg, phosphorous 280 mg, iron 15.2 mg in 100 g of seed. Since the projected food demand for 2025 (Borlaug, 2002) will require yield of millets to rise from 2.5 to 4.5 ton/ha, which will happen through improved varieties modified for resistance to biotic and abiotic stresses (1,2,3). Therefore, there is an urgent need to modify this crop for sustained resistance to abiotic and biotic stresses. Genetic base of the local genotypes is very narrow and attempts for improvement so far have been restricted to pure line selection due to the complicated floral biology of this crop. To meet the strong increase in cereal demand worldwide, new approaches and technologies for generating new varieties are necessary (4,5,6). One of these methods is the creation of transgenic plants with desirable traits.

Monocots in general and cereals in specific were initially difficult to genetically engineer, mainly due to their recalcitrance to *in vitro* regeneration and their resistance to *Agro bacterium*-mediated infection. However, efficient transformation protocols have been later established for the major cereals including rice and maize. But transformation in millet crops is not well standardized and requires more attention. For successful transformation, an efficient regeneration protocol is a necessary stair. Regeneration of any plant from a tiny explants affected by several internal and external factors which governs the biochemical machinery required to switch the pathway of organogenesis. Plant growth regulators, genotypes and explants are major factors which affect regeneration ability of any plant. Attempts to regenerate barnyard millet using indirect organogenesis and somatic embryogenesis were carried out in previous reports but it still require to study the effect of some important factors which influence the regeneration.

Materials and Methods

Plant material

Mature seeds of *E. frumentacea* variety CO-2were dehusked and thoroughly washed under running tap water followed by 4 to 5 washing with distilled water. These seeds were then treated with Tween-20 (2-3 drops) for 20 min with intermittent shaking followed by rinsing with distilled water. Further sterilization of seeds was carried out inside the laminar air flow chamber with different concentration of mercuric chloride for 3. Sterilized seeds were washed thoroughly with sterile distilled water for 5-7 times to eliminate the mercuric chloride.

Callus induction

Seed were inoculated on MS medium fortified with different concentration of 2, 4-D and NAA. In first step these growth regulators were used alone and after preliminary experiments, combinations of 2 , 4-D and BAP were formulated and tested for callus induction ability. On an average 15 mature seeds were inoculated in each plate, sealed with parafilm^{TM} and incubated at a photosynthetic photon of 50µmol^{-2} s^{-1} with 25±2°C under 16/8 hour photo period conditions.

Callus proliferation and maintenance

The resulting calli were subculture on similar medium after 21 days. Well-developed calli were transferred to MS medium with different concentration of BAP and kinetin and combination of both growth regulators. MS medium without any growth regulator was used as control. Sealed plates were incubated at a photosynthetic photon of 50µmol^{-2} s^{-1} with 25±2°C under 16/8 hour photo period conditions. Callus showing shoot induction and shoots induced per explants were counted after one month of incubation.

Shoot induction

Well-developed calli were transferred to MS medium with different concentration of BAP and kinetin and combination of both growth regulators. MS medium without any growth regulator was used as control. Sealed plates were incubated at a photosynthetic photon of 50µmol^{-2} s^{-1} with 25±2°C under 16/8 hour photo period conditions. Callus showing shoot induction and shoots induced per explants were counted after one month of incubation.

Root induction

After shoot induction, individual shoots were separated from the clumps and transferred in test tubes containing MS medium fortified with various types and concentrations of auxins MS medium without auxin and half strength MS medium without auxins were also tested for root induction ability. Root induction percent, root numbers per shoot and root length were recorded after 15 days of incubation at a photosynthetic photon of 50µmol^{-2} s^{-1} with 25±2°C under 16/8 hour photo period conditions.

Plantlet hardening and acclimatization

In vitro rooted shoots were carefully removed from nutrient medium and washed with tap water to remove adhered medium without damaging the roots. A mixture of vermicompost, sand and soil were mixed in combination of 1:1:1 and filled in plastic pots for plantation of rooted plantlets. These were covered with a transparent plastic sheet for 10-12 days and were incubate at 28°C. The cover was gradually removed after twelve days, initially for 3 h followed by 6 h and 12 h at three days intervals. Subsequently, the period of keeping the plantlets without any cover was gradually increased and after 21 days they were brought outside under a shade. Within next 10 days these plants were gradually exposed to sun.

Data analysis

The data was analyzed statistically by simple CRD for callus induction, shoot regeneration and root induction to study the role of growth regulators on in vitro regeneration ability.

Results and Discussion

Callus induction

Results obtained in Callus induction was observed on MS medium either supplemented with 2,4-D alone (1-6 mg/l), lower 2,4-D (2.0 mg/l) with lower BAP (1-2 mg/l) or higher 2,4-D (4 mg/l) combined with higher BAP (4-6 mg/l). Mature seeds were unable to induce callus in MS medium without growth regulators and MS medium fortified with NAA. Initially, seeds germinated abnormally with swollen and short plumule and radical in 2, 4-D

containing medium. Callus of loose and fragile nature was observed on plumule region after 10-12 days of incubation which further spread throughout the seedling. On MS medium without growth regulators and supplemented with NAA, seeds germinated normally. Maximum callus induction (75.56%) was obtained on MS medium fortified with 5 mg/l 2, 4-D.

Shoot induction

Results obtained in shoot induction medium are presented in MS medium supplemented with different concentrations of BAP, maximum shoot induction and shoots per callus obtained on medium fortified with 0.5 mg/l BAP followed by 1 mg/l BAP. Genotype CO2 showed significant maximum shoot induction response (75.41%) on MS medium containing 0.5mg/l BAP. Maximum shoots per callus (5.52) were induced in genotype CO2 on MS medium supplemented with 0.5 mg/l BAP. Results also obtained on MS medium containing different concentrations of kinetin. Maximum shoot induction and numbers of shoots per callus were obtained on MS medium containing 0.5 mg/l kinetin followed by 1 mg/l kinetin.

Table.1 Callus Induction

Callus induction in different media	
Genotype Media	**CO-2 cultivar**
Control	0.00
1D	$55.56 \pm 5.09^{c, x}$
2D	$63.33 \pm 3.33^{c, x}$
3D	$67.78 \pm 1.92^{b, x}$
4D	$68.89 \pm 5.09^{ab, x}$
5D	$75.56 \pm 3.85^{a, x}$
6D	$50.00 \pm 3.33^{ab, z}$
1NAA	0.00
2D1B	$32.22 \pm 6.94^{d, y}$
2D2B	$10.00 \pm 3.33^{f, y}$
2D3B	0.00
2D4B	0.00
2D5B	0.00
2D6B	0.00
3D3B	2.22 ± 1.92^{ef}
4D4B	$12.22 \pm 3.85^{ef,z}$
5D5B	$17.78 \pm 1.92^{de,x}$
6D6B	$11.11 \pm 3.85^{f,y}$

Table.2 Shoot Induction

Genotypes	CO2cultivar.	
Media	Number of calli with shoot induction (%)	Numbers of Shoots per Callus
MS basal	15.56±1.92^{i,x}	1.21±0.23$^{g, xy}$
0.5B	75.41±5.09^{a,x}	5.52±0.16$^{a, xy}$
1B	62.68±6.94^{b,z}	5.28±0.68$^{ab, x}$
1.5B	54.44±1.92b^{cd,z}	4.42±0.16$^{bcd, z}$
2B	51.11±5.09^{cd,y}	4.31±0.07$^{bcd, y}$
0.5K	57.78±5.09^{bc,x}	4.43±0.18$^{bcd, z}$
1K	43.33±5.77^{ef,z}	4.59±0.33$^{bc, y}$
1.5K	37.78±5.09^{f,y}	4.37±0.15$^{bcd, y}$
2K	36.67±5.77^{fg,y}	4.26±1.05$^{cd, y}$
0.5B 0.5K	25.56±3.85^{h,z}	2.57±0.52$^{f, x}$
1B1K	58.89±3.85^{bc,x}	3.25±0.14$^{ef, xy}$
1.5B1.5K	46.67±6.67^{de,x}	3.67±0.88$^{de, z}$
2B2K	28.89±3.85^{gh,yz}	2.71±0.47^{f}

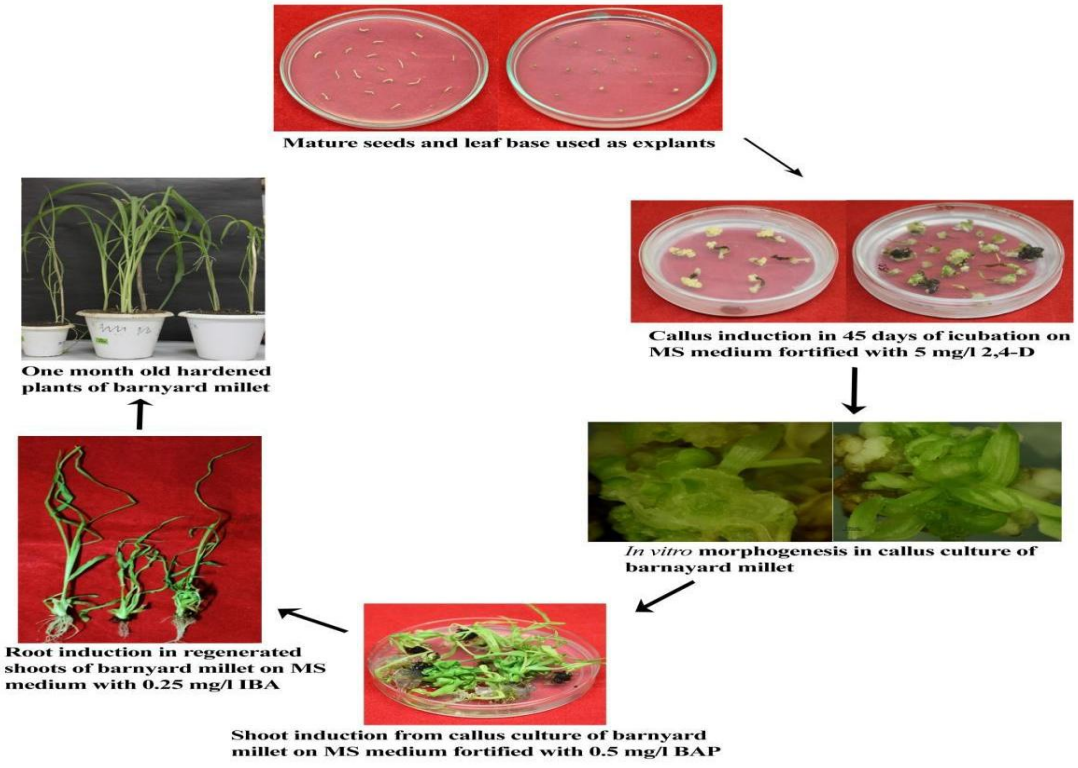

Fig. 1- Development of indirect regeneration protocol of barnyard millet

Table.3 Root induction

Media	Root induction response (%)	Numbers of Roots /shoot	Average root length (cm)
MS Basal	84.81±0.64	4.43±0.97^{e}	3.50±0.85^{c}
Half MS	90.37±1.70	5.60±1.07^{d}	4.38±0.97^{b}
0.25 NAA	83.22±1.92	8.29±0.95^{b}	1.89±0.74^{ef}
0.5NAA	86.67±1.92	7.75±1.03^{c}	1.67±0.70^{f}
0.25 IAA	84.85±3.57	8.12±1.52^{b}	2.64±0.84^{e}
0.5 IAA	86.30±0.64	6.03±1.05^{d}	3.11±0.74^{d}
0.25 IBA	87.04±1.28	10.76±1.49^{a}	5.39±0.70^{a}
0.5 IBA	86.30±2.31	10.37±1.35^{a}	5.08±0.67^{a}

Root induction

Significant effect of root induction medium on numbers of roots per shoot and average root length was observed during present investigation. MS medium supplemented with 0.25 mg/l and 0.5 mg/l IBA exhibited significantly maximum roots per shoot (10.76) and (10.36) followed by MS medium fortified with 0.25 mg/l NAA (8.29) and MS medium fortified with 0.25 mg/l IAA (8.12). Similarly, MS medium supplement with 0.25 mg/l and 0.5 mg/l IBA exhibited significant highest average root length (5.39) and (5.08) followed by half strength MS medium without growth regulator (4.38). MS medium supplemented with NAA showed significant lowest average root length among all root induction medium tested.

It is concluded in the present investigation, a 90 days regeneration protocol has been established for barnyard millet cultivar CO2. In conclusion, an easy, efficient and reproducible regeneration protocol was developed to speed up the process of in vitro plant regeneration. The plants produced by this protocol displayed no significant differences in morphology, growth characteristics and survival with the normal plants developed via seed germination. The protocol optimized here can prove to be an efficient for the incorporation of agronomic ally important traits into locally adapted genotypes across the barriers of incompatibility.

References

1. Borlaug NE. 2002. Feeding a world of 10 billion people: The miracle ahead. *In Vitro* Cellular and Developmental Biology-Plant 38:221-228.
2. Doggett H. 1989. Small Millets: A selective Overview. In: Seetharam A, Riley K and Harinaryana G. (eds.): Small Millets in Global Agriculture. Oxford and IBH, New Delhi. 3-18.
3. Dosad and Chawla. 2015. Optimized different concentrations of growth regulators for *in vitro* regeneration of *Echinochloa frumentacea* Roxb. from caryopsis. Indian Journal of Plant Physiology 20(4): 339-344.
4. Gaspairs S, Bregire C, Orczyk W and Nadolska-Orczyk A. 2008. Agrobacterium mediated transformation of oat (*Avena sativa* L.) cultivars via immature embryo and leaf explants. Plant Cell Reports 27:1721-1729.
5. GR Rout, Samantary S and Das P. 1998.

Regeneration of a metal tolerant grass *Echinochloa colona* via somatic embryogenesis from suspension culture. Biologial Plantarum 40(1):17-23.

6. Jagga-Chugh S, Kachhwaha S, Sharma M, Kothari-Chajer A and Kothari SL. 2011. Optimization of factors influencing micro projectile bombardment-mediated genetic transformation of seed-derived callus and regeneration of transgenic plants in *Eleusine coracana* (L.) Gaertn. Plant Cell Tissue and Organ Culture 109:401-410.
7. Kothari SL, Agarwal K and Kumar S. 2004. Inorganic nutrient manipulation for highly improved *in vitro* plant regeneration in finger millet - *Eleucine coracana* (L.) Gaertn. *In vitro* Cellular Development Biology Plant 40:515-519.
8. Kothari SL, Kumar S, Vishnoi RK, Kothari A and Watanabe KN. 2005. Applications of biotechnology for improvement of millet crops: Review of progress and future prospects. Plant Biotechnology 22:81-88.
9. Samantaray S, Rout GR and Das P. (1997). Regeneration of plants via somatic embryogenesis from leaf base and leaf tip segments of *Echinochloa colona* Plant Cell Tissue and Organ Culture 47:119-125.
10. Seetharam, A. 2011. Small millets as viable crops - recent developments, challenges and research gaps. URL:http://www.dhan.org/smallmillets/ presentations.php
11. Skoog F and Miller CO. 1957. Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symposia of the Society for Experimental Biology 11:118-131.
12. Srivastav S and Kothari SL. 2002. Embryogenic callus induction and efficient plant regeneration in pearl millet. Cereal Research Communications 30:69-74
13. Stasolla C and Yeung EC. 2003. Advances on embryogenesis in culture of coniferous species: improving somatic embryo quality. Plant Cell Tissue and Organ Culture 74:15-35.
14. Vasil V and Vasil IK. 1981. Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum*, and *P Americanum* x *P. purpureum* hybrid. American Journal of Botany 68:864-872.
15. Yabuno T. 1987. Japanese Barnyard Millet (*Echinochloa utilis*, Poaceae) in Japan. Economic Botany 41(4): 484-493.
16. Yemets AI, Bayer GY and Blume YB. 2013. An Effective Procedure for *In Vitro* Culture of *Eleusine coracana* (L.) and Its Application. International Scholarly Research Notices Botany 853121.