

Original Research Article

To Study the Indirect Plant Regeneration of Two Cultivars in Barnyard Millet using Different Combination of Plant Growth Regulators and Compared between Superior Genotype for *in-vitro* Culture`

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ABSTRACT

Keywords

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An efficient somatic embryogenesis and plant regeneration system was developed from mature seed explants of barnyard millet two genotypes, CO2 and PRJ1 were assessed in this study. The maximum somatic embryogenic induction, at 75% and 65% respectively, was obtained from explants cultured on Murashige and Skoog medium supplemented with 5D, 2, 4-dichlorophenoxyacetic acid. The highest number of shoot induction was observed after transfer of embryonic callus to regeneration medium supplemented with 0.5mg/1BAP and the highest number of root induction found in half MS medium significant differences were observed between genotypes for somatic embryogenesis and plant regeneration. CO2 gave the best response, while PRJ1 was the least responsive under the culture conditions tested in this study. Regenerated plants were successfully rooted and grown to maturity after hardening in soil.

Introduction

Barnyard Millets represent the small-seeded group of the Poaceae family. The similarities of millets are that they are grown under extreme environmental conditions and therefore, especially suited to areas with inadequate moisture or short-growing cycle and poor soil fertility (Baker, 2003). Barnyard Millets play key role in the maintenance of food security in the developing world since they are the major food and feed sources. Millets are rich sources of human and livestock nutrition in developing countries (NAS, 1996). 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). 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 (Jagga-Chugh *et al.*, 2011). 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. . Regeneration of any plant from a tiny explant 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-2 and PRJ1 were 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 parafilmTM and incubated at a photosynthetic photon of 50 μ mol-2 s⁻¹ with 25 \pm 2 $^{\circ}$ 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 50 μ mol-2 s⁻¹ with 25 \pm 2 $^{\circ}$ 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 $50\mu\text{mol}^{-2}\text{ s}^{-1}$ with $25\pm 2^\circ\text{C}$ 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 $50\mu\text{mol}^{-2}\text{ s}^{-1}$ with $25\pm 2^\circ\text{C}$ 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 (Table 1).

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 found in CO2 cultivar (75.56%) and (68.89%) PRJ1 cultivar 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%) and respectively PRJ1 cultivar (67.78%) on MS medium containing 0.5mg/l BAP. Maximum shoots per callus show in CO2 cultivar (5.52) and PRJ1 cultivar show highest (5.79) were induced in MS medium supplemented with 0.5 mg/l BAP. results also obtained on MS medium containing different concentrations of kinetin (Table 2).

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.

Table.1 Callus induction % in CO2 and PRJ1 cultivar

Callus induction in different media		
Genotype Media	CO-2 cultivar	PRJ1
Control	0.00	0.00
1D	55.56 ± 5.09 _{c, x}	48.89 ± 8.39 _{d, y}
2D	63.33 ± 3.33 _{c, x}	58.89 ± 1.92 _{bc, y}
3D	67.78 ± 1.92 _{b, x}	58.89 ± 5.09 _{bc, y}
4D	68.89 ± 5.09 _{ab, x}	63.33 ± 3.33 _{b, y}
5D	75.56 ± 3.85 _{a, x}	68.89 ± 5.09 _{a, y}
6D	50.00 ± 3.33 _{ab, z}	55.56 ± 1.92 _{c, x}
1NAA	0.00	0.00
2D1B	32.22 ± 6.94 _{d, y}	0.00
2D2B	10.00 ± 3.33 _{f, y}	0.00
2D3B	0.00	0.00
2D4B	0.00	0.00
2D5B	0.00	26.67 ± 3.33 _{e, z}
2D6B	0.00	11.11 ± 5.09 _{g, x}
3D3B	2.22 ± 1.92 _{ef}	0.00
4D4B	12.22 ± 3.85 _{ef, z}	0.00
5D5B	17.78 ± 1.92 _{de, x}	0.00
6D6B	11.11 ± 3.85 _{f, y}	0.00

Table.2 Shoot induction and shoot per callus % in CO₂ and PRJ1 cultivar

Genotypes	CO ₂ cultivar.		PRJ1 cultivar	
	Number of calli with shoot induction (%)	Numbers of Shoots per Callus	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}	15.56±3.85 _{f,x}	1.37±0.15 _{h, x}
0.5B	75.41±5.09 _{a,x}	5.52±0.16 _{a, xy}	67.78±1.92 _{a,z}	5.79±0.28 _{a, x}
1B	62.68±6.94 _{b,z}	5.28±0.68 _{ab, x}	61.11±1.92 _{ab,xy}	5.15±0.28 _{abc, xy}
1.5B	54.44±1.92 _{bcd,z}	4.42±0.16 _{bcd, z}	58.89±5.09 _{ab,x}	4.62±0.55 _{cd, xy}
2B	51.11±5.09 _{cd,y}	4.31±0.07 _{bcd, y}	54.44±6.94 _{bcd,x}	4.22±0.43 _{de, yz}
0.5K	57.78±5.09 _{bc,x}	4.43±0.18 _{bcd, z}	46.67±3.33 _{d,z}	5.62±0.60 _{ab, x}
1K	43.33±5.77 _{ef,z}	4.59±0.33 _{bc, y}	48.89±5.09 _{cd,x}	4.36±0.69 _{cde, z}
1.5K	37.78±5.09 _{f,y}	4.37±0.15 _{bcd, y}		
			54.44±8.39 _{bcd,x}	3.62±0.24 _{ef, z}
2K	36.67±5.77 _{fg,y}	4.26±1.05 _{cd, y}	47.78±10.18 _{cd,x}	3.57±0.90 _{ef, z}
0.5B 0.5K	25.56±3.85 _{h,z}	2.57±0.52 _{f, x}	28.89±5.09 _{e,y}	2.32±0.41 _{g,y}
1B1K	58.89±3.85 _{bc,x}	3.25±0.14 _{ef, xy}	56.67±3.33 _{bc,y}	3.38±0.31 _{f, xy}
1.5B1.5K	46.67±6.67 _{de,x}	3.67±0.88 _{de, z}	33.33±3.33 _{e,z}	4.86±0.07 _{bcd, x}
2B2K	28.89±3.85 _{gh,yz}	2.71±0.47 _f	27.78±3.85 _{yz}	2.96±0.17 _g

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

Pictures of callus and shoot induction in two cultivars



CALLUS AND SHOOT INDUCTION IN CO2



CALLUS AND SHOOT INDUCTION IN PRJ1 CULTIVARS

In the present investigation, a 90 days regeneration protocol has been established for barnyard millet cultivar CO2 and PRJ1. In conclusion, an easy, efficient and reproducible regeneration protocol was developed to speed up the process of *in vitro* plant regeneration. And obtained CO2 cultivar is superior to PRJ1 cultivar in compared callus induction and shoot induction % but PRJ1 cultivar show highest shoot per callus as well as the ratio were found 2:1 between two cultivar of barnyard millet. 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 agronomically

important traits into locally adapted genotypes across the barriers of incompatibility.

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