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Efficient Plant Regeneration from Shoot Apices of Pearl Millet (*Pennisetum americanum* (L.) Leeke)

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Abstract

An efficient and rapid regeneration protocol was developed from shoot tip explants of *Pennisetum americanum* (L.) Leeke variety WC-75. MS supplemented with low concentration of 2,4-D and Kn was most effective in producing embryogenic calli. Maximum regeneration potential of 40 shoots per calli were obtained when transferred to regeneration medium containing 1.0 mg/l Kn. Shoots developed were efficiently rooted within 15 days on the medium containing NAA. Over 90% of rooted plants were fertile after transfer to a net house.

Introduction

Pearl millet (*Pennisetum americanum* (L.) Leeke) is an important staple food grain in India and Western Africa and is widely cultivated in semi-arid regions especially under low soil fertility and harsh climatic conditions. Pearl millet is highly susceptible to downy mildew disease caused by the fungus *Sclerospora graminicola* (Sacc.) Schroet., which can cause up to 70% reduction in yield. Insect pests also inflict severe damage in field and during storage. Therefore, improvement of pearl millet crop will have a great effect on socio-economic status of the people in rural areas (Arockiasamy et al. 2001), where it is widely cultivated. Traditional breeding has been the main avenue for crop improvement in pearl millet until recently. The objectives of improvement include better utilization of natural resources, development of resistance against biotic and abiotic stresses and improvement of quality for wider consumer acceptance. However, improvement of millets using biotechnology has largely been unattempted, due not only to economic or regional considerations but also non-availability of robust and reproducible regeneration protocols (Kothari et al. 2005). An efficient regeneration system is often a pre-requisite for application of

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biotechnological applications like gene transfer. Successful regeneration in pearl millet has been reported from immature embryos (Vasil and Vasil 1982) and immature inflorescences (Pius et al. 1993, Mythili et al. 1997). However, production of these explants is a space, time and labour consuming task. Explants such as mature embryos from seed (Botti and Vasil 1983), shoot apices or meristems (Mythili et al. 2001, Devi et al. 2000) are definitely more convenient. Reports of successful callus induction and plant regeneration from shoot apices in majority of cereal crops, including maize (Zhong et al. 1992), wheat (Ahmad et al. 2002), rice (Nhut et al. 2000), barley (Ganeshan et al. 2003), sorghum (Maheswari et al. 2006) and millets (Devi et al. 2000) provide evidence for developmental plasticity of the apical shoots. Shoot apical meristems of cereals are also easily amenable for shoot regeneration, transformation studies and is highly advantageous for rapid regeneration into mature plants (Sticklen and Oraby 2005).

In this paper, we report a highly efficient, rapid season-independent protocol for plant regeneration from shoot apices of pearl millet.

Materials and Methods

Seeds of pearl millet (*Pennisetum americanum*) (L.) Leeke cv. WC-75 were obtained from Dr. K.N. Rai, ICRISAT (Patancheru, Andhra Pradesh). Surface sterilization of the seeds was done by rinsing them in 70% alcohol for one min followed by 0.1% mercuric chloride for two min. The seeds were then washed in sterile distilled water five times and were aseptically germinated on wet cotton in culture bottles (50 seeds per bottle) in the dark at 25ºC. After 48 hrs of germination, 3 mm sections of shoot apices were carefully dissected from the germinating seedlings. The sections were then cultured on MS supplemented with various concentrations of 2,4-D (0.50, 0.75, 1.0, 2.0 and 3.0 mg/l) and Kn (0.25 and 0.50 mg/l) and solidified with 0.8% agar. Ten explants were horizontally placed in the medium in each Petri dish and were maintained in dark at 25 ± 1ºC. Callus initiation was observed typically ten days after inoculation. Culture responses were recorded, based on the callus diameter and fresh weight. The calli were subcultured at 13 days intervals and at the time of each transfer leaves, coleoptiles and elongated shoots were continuously removed from the explant so as to expose the shoot apices. Twenty five days after inoculation cultures were transferred to regeneration medium containing Kn (1.0 mg/l). The cultures were then grown in light (85 μmol/m^2/s) under a 12 hrs photoperiod for 20 days. The relative efficiency of multiple shoot regeneration was evaluated by visually scoring the shoots 15 days after sub-culturing. Regeneration frequency was calculated based on the ratio of calli showing shoot emergence to the total number of calli in percentage. Regenerated

plantlets were then transferred to rooting medium containing NAA (1 mg/l) and maintained for 15 days. Subsequently plantlets with well-developed root system were transferred to a substrate comprising 1 : 1 peat and soil rite for further development in green house. Each treatment had three replications and each experiment was repeated at least twice.

Results and Discussion

Initial culturing of the shoot apex explants on MS containing 2,4-D in combination with Kn resulted in creamish white calli (Fig. 1a). Subsequently embryogenic, compact, nodulated callus, developed by two weeks (Fig. 1b). Induction of embryogenic callus was also reported at a 2, 4-D concentration of around 2.0 mg/l (Vasil and Vasil 1981, Taylor and Vasil 1991, O' Kennedy et al. 2004). No callusing response was observed when explants were cultured on MS without any plant growth regulators.

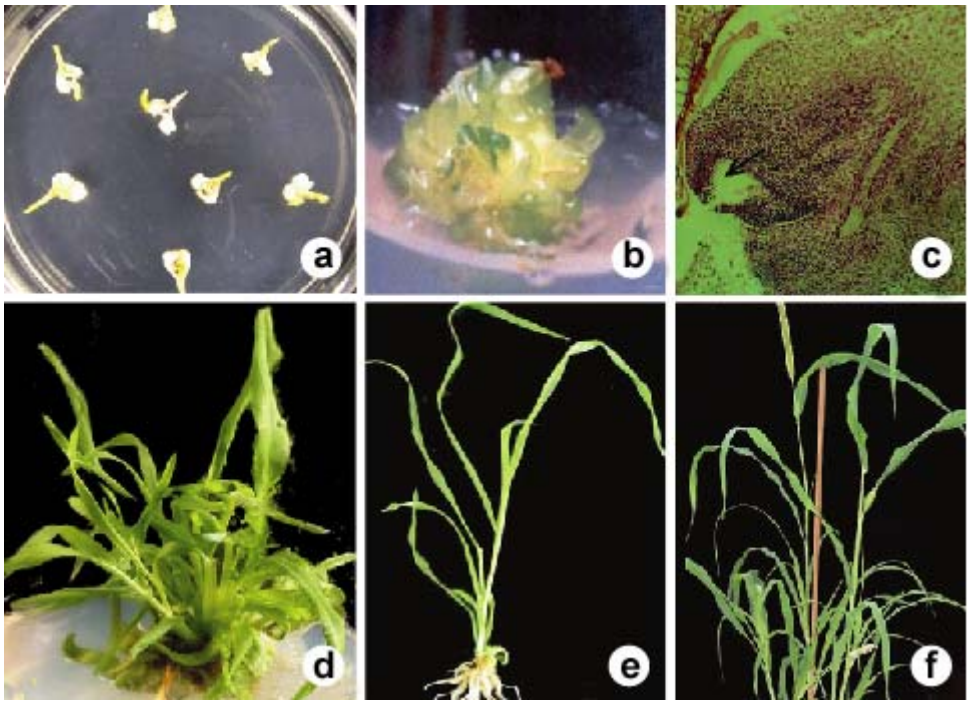

Fig. 1. Shoot regeneration from apical meristems in *P. americanum.* (a). Explants on the callus induction medium. (b). Twenty five days old calli on induction medium. (c). *De novo* formation of shoot buds at initial stage of development. (d). Regeneration of shoots. (e). Rooting (f). Regenerated plants growing in pot.

In general, the callus induction frequency was 100% in MS containing 2,4-D in combination with 0.25 mg/l Kn, while it ranged from 85 - 95% on all other media combinations tested. The largest callus diam (0.93 cm) and maximum

fresh weight (0.50 g) of calli were recorded on 2,4-D at 0.5 mg/l concentration in combination with 0.25 mg/l Kn, compared to all other combinations (Table 1). But it is to be noted that this high concentration of 2,4-D in particular led to complete inhibition of shoot regeneration at a later stage when cultured on regeneration medium.

Table 1. Effect of 2,4-D and Kn on callus induction in shoot tip derived cultures of pearl millet cv. WC-75.

Growth regulators (mg/l)		Callus diam (cm)	Fresh wt. of callus (g)
2,4-D	Kn		
0.50	0.25	0.93 ± 0.01	0.503 ± 0.03
0.50	0.50	0.83 ± 0.02	0.401 ± 0.02
0.75	0.25	0.73 ± 0.01	0.436 ± 0.04
0.75	0.50	0.76 ± 0.03	0.461 ± 0.04
1.00	0.25	0.80 ± 0.00	0.464 ± 0.02
1.00	0.50	0.73 ± 0.01	0.416 ± 0.02
2.00	0.25	0.76 ± 0.01	0.388 ± 0.01
2.00	0.50	0.90 ± 0.01	0.383 ± 0.04
3.00	0.25	0.80 ± 0.01	0.465 ± 0.04
3.00	0.50	0.70 ± 0.01	0.411 ± 0.05

Data on all the parameters were recorded two weeks after culture.

Light microscopic observation of 25 days old callus growing on regeneration medium clearly revealed initiation of *de novo* shoot buds from callus cultures (Fig. 1c).

Inclusion of Kn in callus induction medium facilitated callus growth as well as subsequent shoot regeneration. Regeneration response of the calli obtained on different combinations of 2,4-D and Kn was tested on 1 mg/l Kn. Combination of BAP (4.43 μM) and Kn (4.64 μM) has also been reported to enhance shoot regeneration in *P. glaucum* (Arockiasamy et al. 2006). In present experiments, 2,4-D concentrations of 0.5, 0.75 and 1.0 mg/l in combination with lower concentrations of Kn were very effective in obtaining a good regeneration frequency and number of shoots (Table 2). 2,4-D at a concentration of 0.75 and 0.25 mg/l Kn showed highest regeneration frequency of 62.5% as well as shoot number of 40 shoots (Fig. 1d). The callus induction frequency was also 100% in the above mentioned concentration. Increased concentration of 2,4-D led to a lower regeneration frequency and shoot number. The presence of 2,4-D in the medium was definitely detrimental to chlorophyll synthesis in callus (Bhaskaran et al. 1992). Kn was able to restore chlorophyll content in callus to a certain

extent. Thus at higher concentrations of 2,4-D in combination with Kn a moderate regeneration response was observed. Kn was shown to be more effective in improving the regeneration potential.

Table 2. Effect of 2,4-D and Kn on shoot tip derived calli on transferring to regeneration medium supplemented with Kn (1.0 mg/l) after four weeks of culture.

Growth regulators (mg/l) in callus induction medium		No. of shoots per callus
2,4-D	Kn	
0.50	0.25	53.5 ± 1.50
0.50	0.50	46.0 ± 1.00
0.75	0.25	80.0 ± 2.00
0.75	0.50	66.0 ± 3.00
1.00	0.25	54.0 ± 1.00
1.00	0.50	62.5 ± 3.00
2.00	0.25	47.0 ± 5.00
2.00	0.50	41.0 ± 5.00
3.00	0.25	40.0 ± 2.00
3.00	0.50	37.0 ± 2.00

After 20 days on regeneration medium, shoot formation was observed. Shoots were green and healthy. Individual shoots were separated and transferred to the medium containing NAA (1.0 mg/l) for rooting. Roots developed within 15 days (Fig. 1e). Over 90% of rooted plantlets grew well and were fertile after transfer to the net house (Fig. 1f). The developmental morphology of these plants was similar to that of seed derived plants in the net house and they flowered normally and produced mature viable seeds.

An efficient and rapid plant regeneration protocol for pearl millet using shoot apicesis is reported here. The morphogenetic response of the shoot apices depended on the concentration of 2,4-D and Kn in present experiments. Lower concentrations of 2,4-D and Kn (0.75, 0.25 mg/l) were very effective in producing embryogenic calli which upon transfer to a higher Kn (1.0 mg/l) containing medium clearly improved regeneration efficiency with nearly 40 shoots per callus. Here, regeneration system is also relatively rapid as the regenerated and rooted plantlets can be obtained within 65 - 67 days. This kind of a robust regeneration system is critical for efficient genetic transformation of pearl millet.

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