J. Phytol. Res. **13** (2) : 153-156, 2000

MICROPROPAGATION AND *IN VITRO* FLOWERING FROM TEN-DAY-OLD ZYGOTIC EMBRYOS OF PEARL MILLET (*PENNISETUM GLAUCUM* (L.) R. BR.)

T.V.R. LAKSHMI, K. ARUNA LAKSHMI, K.G. RAJA RAO and P.S.R.L. NARASINGA RAO
Department of Botany, Andhra University, Waltair - 530 003, India.

Two distinct protocols have been identified; in which plantlets were obtained form ten day old zygotic embryos of four cultivars of pearl millet. In the first protocol a clear role for 2, 4-D was noticed. The zygotic embryos cultured on MS+2,4-D medium gave rise to embryogenic calli in 2 weeks and plantlets in 4 weeks on 2,4-D free MS medium. In the second protocol, "hormone-addition" had the effect of multiple shooting and *in vitro* flowering in the germinated seedlings. The zygotic embryos cultured on MS+0.5 NAA+0.5 BAP (mg/l each) germinated into healthy seedlings of which 20% were with multiple axillary shoots. *In vitro* flowering was obtained from multiple axillary shoots alone though in a low frequency (6.6%). This phenomenon is repeatable.

Keywords : *In vitro* flowering; Multiple axillary shoots; Pearl millet; Plant regeneration; Zygotic embryos.

Introduction

Pearl millet, *Pennisetum glaucum* (L.) R.Br. is among the most nutritious grain crops due to its high and good quality protein. Plant regeneration in this species has been achieved from mature and immature embryos[1,2], mesocotyl[3], immature inflorescence[4] and protoplasts[2]. Attempts are now being made to improve the varieties of pearl millet through genetic transformations that would resist from major diseases like downy mildew, ergot and smut, causing considerable damage. Recent studies revealed that immature zygotic embryos serve as potential tools for genetic transformation in rice[5] and maize[6]. A prerequisite for these transformations is a routine plant regeneration protocol.

The aim of the present investigation is to devise reproducible protocols for micropropagation and *in vitro* flowering respectively from ten-day-old zygotic embryos of pearl millet. Although a substantial number of reports on regeneration in pearl millet are available, an effective, plantlet formation in a short time besides flowering is being reported for the first time.

Materials and Methods

Ten-day-old zygotic embryos were chosen as explants in the present investigation due to their greater responsiveness over the mature embryos[7]. Developing caryopses (10 days after pollination) were excised from the control pollinated ear heads. The excised caryopses were surface sterilized with 0.01% $HgCl_2$ for 15 minutes and washed with sterile water several times, and the zygotic embryos were dissected out. Four cultivars viz., Vg 272 (a local variety), ICP 501 (a wild variety with awned ear heads), IP 8182 (a purple pigmented variety) and Upper Volta SL-2 (containing B-Chromosomes in addition to normal Chromosomes) of Indian and African origin were selected for the present investigation. The local cultivar Vg 272 has been selfed for more than 20 generations , while the seeds of the other varieties are suppllied by the ICRISAT.

MS medium supplemented with 1.0 mg /l 2, 4-D is optimum for the induction of embryogenic calli in Vg 272[7]. Hence, the embryos of the 4 cultivars were cultured on MS[8] + 1.0 mg/1 2, 4-D for callus initiation and subsequently on 2,4-D free MS medium for germination of somatic embryos into plantlets.

Micropropagation and in vitro flowering : The zygotic embryos were cultured on MS+0.5 NAA+0.5 BAP (mg/1 each), which after 10 days were transferred to MS+0.4 mg/1 NAA + 1.0 mg/1 BAP. MS medium supplemented with different concentrations of NAA and BAP were tested for *in vitro* flowering. The media were supplemented each with 2% sucrose and 0.8% agar. All the cultures except those kept for regeneration were maintained at 28±2°C with 8/16 h light/ dark photoperiod while those for regeneration

were under continuous light. The multiple shoots were acclimatised in Hoagland's[9] nutritive solution before the plants were transferred to soil.

Results and Discussion

Callusing and regeneration : Callusing was visible on the third day after inoculation from the zygotic embryos of the four genotypes cultured on 2,4-D medium. All the embryos of IP 8182 responded while a few of these in other three varieties remained dormant (Table 1). The initial callus was friable, glistening and pearly white with purple islands here and there in IP 8182, while the calli of three other lines were pearly white. An embryogenic, compact nodulated callus appeared in about 2 weeks after inoculation in all the cultivars (Fig 1). The embryogenic callus upon subculture on MS medium differentiated into plantlets. Each embryogenic callus gave rise to 2-15 plantlets (Fig 2). The response of the embryos could be classified into least, moderate and high (Table 1).

Germination of zygotic embryos : The zygotic embryos cultured on NAA+BAP (0.5 mg/1 each) medium germinated by the third day with scanty mesocotyl callusing among the four cultivars. It was observed that the genotype Vg 272 was most responsive and ICP 501 the least (Table 1). The seedlings of IP 8182 were purple while those of other cultivars were green.

In Vg 272, among the germinated seedlings, 80% of the embryos developed into single shoots while the remaining 20% produced multiple axillary shoots (Fig 3). Multiple shoot formation was more or less similar in all the 4 cultivars of the present study. The multiple shoots were separated one from another and upon their transfer to MS+1.0 mg/1 NAA, rooting was observed.

In vitro flowering in Vg 272 : The seedlings with single and multiple shoots after 10 days of culturing were transferred to MS+0.4 mg/1 NAA + 1.0 mg/1BAP. The single shoots did not bolt/produce any inflorescence while a few multiple shoots did in about 5-6 weeks. The seedlings with more (about 10) shoots flowered (Fig 4) while those with fewer failed to do so. The inflorescence was of an inch long after it fully emerged from the boot. About 10 spikelets were borne on each inflorescence. Each spikelet contained normal looking glumes, anthers and an ovary. The stigmas emerged prior to the anther dehiscence resembling the protogynous nature of *in vivo* flowers. Versatile white coloured anthers dehisced a pair each day.

Protocol for *in vitro* flowering in pearl millet. (Hormone conc. in mg/l)

10 day old embryos
↓
on MS+0.5 NAA+0.5 BAP(medium 1)
↓ for 10-days
MS+0.4 NAA+1.0 BAP.(medium 2)
↓ for 30-40 days
↓
Multiple shoots flowered.

Table 1. Response of 10 days old zygotic embryos to callusing, regeneration and germination media in the four cultivars of pearl millet.
(MS+Hormone conc. in mg/l+0.8% agar), cultures maintained at 28±2°C; 8/16 h light/dark photoperiod.

S. No.	Callusing and regeneration medium MS + 1.0 2, 4-D (mg/l)				Germination medium MS + 0.5 NAA+0.5 BAP (mg/l)	
	Cultivar	No. zygotic Embryos cultured	% callused	% regenerated	No. zygotic Embryos cultured	% germinated
1.	Vg 272	127	99.3	70.0	105	100
2.	IP 8182	50	100.0	59.0	54	74.0
3.	ICP 501	55	41.8	28.5	80	42.5
4.	SL - 2	63	77.7	46.6	51	47.0

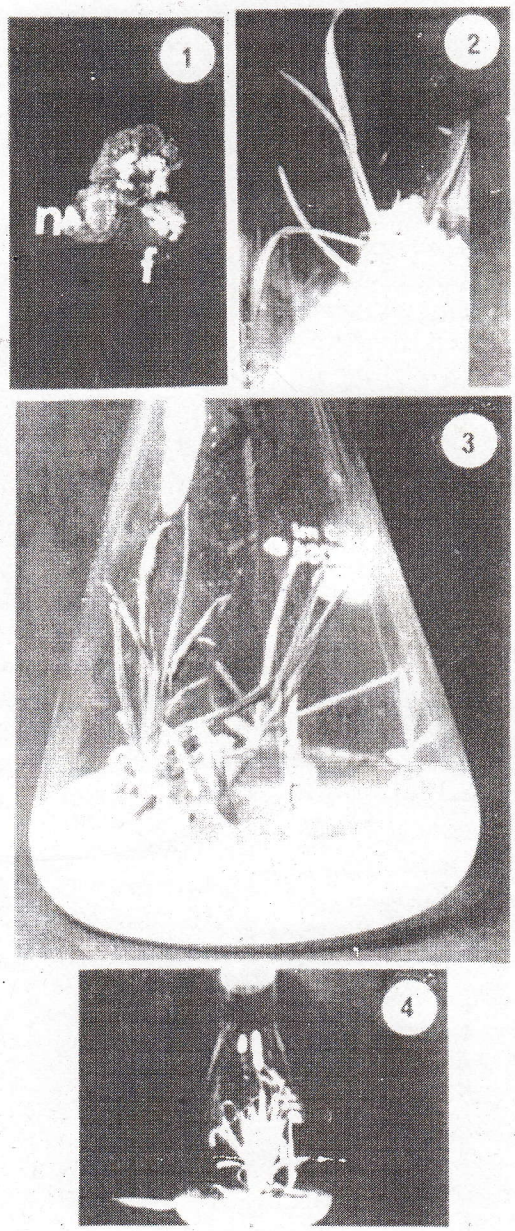

Fig. 1,2,4. Cultures of Cultivar Vg 272
Fig. 3. Cultures of Cultivar ICP 501

Fig. 1. Embryos showing nodulated embryogenic callus (n) on a carpet of friable callus (f) on MS + 1.0 (mg/l) 2, 4-D medium
Fig. 2. Plantlet formation from 3-week old embryogenic callus on MS basal medium.
Fig. 3. Multiple axillary shoots from 2 embryos on MS + 0.5 NAA+0.5 BAP (mg/l)
Fig. 4. *In vitro* flowering from multiple axillary shoots on MS + 0.4 NAA + 1.0 BAP (mg/l)

Medium 1 or medium 2 alone did not induce *in vitro* flowering in either multiple or single shoots. Flowering was observed among the multiple shoots of the other three cultivars when subcultured on medium - 2 from medium-1, however detailed study was carried only in Vg 272.

The present study indicates that 2,4-D encouraged callus formation and suppressed seedling growth, while combination of NAA + BAP allowed gemination of the zygotic embryos. The four cultivars of pearl millet responded similarly to callusing medium and germination medium. Thus, in pearl millet, the embryo response can be controlled by the type of hormone in the medium. However, the differences in response of embryos to each of the media among the four cultivars may be attributed to the varietal difference. The differences in the response of the embryos to each medium in a single cultivar can be accounted for the heterogeneity in that cultivar.

Induction of multiple axillary shoots through the combination of NAA and BAP is being reported in the four cultivars of pearl millet for the first time. Axillary shoot proliferation was reported in rice[10] and wheat[11] which are self pollinated crops. It was induced by BAP in rice[10] and zeatin or kinetin in wheat[11]. Pearl millet being an open pollinated crop, also forms multiple axillary shoots in the medium containing NAA+BAP. However, it differs from rice and wheat in that only a combination of NAA and BAP induces multiple shooting (T V Rajya Laksmi, unpublished). Thus, this technique can also be used for micropropagation of cultivars whose regeneration potential is low and also for inter-specific hybrids.

In vitro flowering was induced in four cereals viz. maize[12], wheat[11], triticale[13] and pearl millet[14]. It was reported that flowering in cultures was as an unusual, abnormal or one time phenomenon in these cereals including pearl millet. In the present study, induction of flowering, as and when desired could also be achieved as subculture of multiple axillary shoots in decreased auxin and increased cytokinin led to flowering *in vitro* in the four cultivars of pearl millet. However, the protocol standardised in the present investigation is not abnormal or unusual or one-time phenomenon, but repeatable as in Bamboos[15], producing protogynous florets. Interestingly *in vitro* flowering is observed only in multiple shoots and not in single shoots. Multiple shoots flowered in about 2 months rather than after 3 to 4 months as is customary for normal field grown plants. Further studies in this direction would unravel the events/factors underlying the switch from vegetative to flowering state. As gametes could be obtained in sterile condition, extension of this technique to other varieties is useful. This data helps us to induce multiple shoots and subsequently flowering at will.

Acknowledgements

The article is dedicated to Prof. M. Krishna Rao who initiated research in this area. Drs. T.V.R.L. and K.A.L. are thankful to the Council for Scientific and Industrial Research for the award of Research Associateships.

References

1. Botti C and Vasil I K 1983, *Z. Pflangenphysiol* **111** 319
2. Vasil V and Vasil I K 1981, *Am. J. Bot.* **68** 864
3. Subba Rao M V and Nitzsche W 1983, *Euphytica* **33** 923
4. Talwar M and Rashid A 1990, *Ann. Bot.* **66** 17
5. Chan MT, Chang H H, Ho S L, Tong W F and Yu S M 1993, *Plant Mol. Biol.* **22** 491
6. Schlappi M and Hohn B 1992, *Plant cell* **4** 7
7. Lakshmi T V R, Aruna Lakshmi K, Raja Rao K G and Krishna Rao M 1999, *Visakha Sci. J.* **3** 131
8. Murashige T and Skoog F 1962, *Physiol. Plant.* **15** 473
9. Hoagland D R and Arnon D I 1938, In : *Introductory Plant Physiology*, G R Noggle and G J Fritz (eds). Prentice-Hall publisher, India, p 239.
10. Greco B, Lomonaco A, Boggini B, Tomassini C and Tanzarella O A 1990, *Euphytica* **48** 123
11. Tanzarella O A and Greco B 1985, *Euphytica* **34** 273
12. Raman K, Walden D B and Greyson R I 1980, *Ann. Bot.* **45** 183
13. Reddy V D and Reddy G M 1988, *Genet. Agraria.* **42** 151
14. Kulkarni V M, Chaudhari A N, Shah B S and Harinarayana G 1995, I. J. Expt. Biol. 33 142
15. Nadgauda R S, Parasharami V A and Mascarenhas A F 1990, *Nature* **344** 335