

**PLANT REGENERATION BY SOMATIC EMBRYOGENESIS
FROM CALLUS OF MATURE SEED EXPLANTS OF
BREAD WHEAT (*TRITICUM AESTIVUM* L.)**

**SAAD IMRAN MALIK¹, HAMID RASHID², TAYYABA YASMIN
AND NASIR MAHMOOD MINHAS**

*Department of Plant Breeding & Genetics,
University of Arid Agriculture, Rawalpindi, Pakistan.*

Abstract

The objective of this study was to test genotypes and to evaluate media for effectiveness in promoting whole plant regeneration by somatic embryogenesis in two wheat genotypes viz., Inqilab-91 and Pavon-76. Embryogenic callus was obtained by culturing seeds of both varieties on gel-solidified Linsmaier and Skoog's (LS) medium with 3.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The callus cultures were maintained by subsequent subcultures onto fresh media at 4-5 weeks interval. The embryogenic calli carefully cleared of any surrounding non-embryogenic callus were grown on the gel-solidified basic Murashige and Skoog's (MS) medium with 3% (w/v) sucrose after at least two subcultures. Seven different combinations of BAP (6-benzylaminopurine) and IAA (Indole-3-acetic acid) i.e., 0 & 0.1, 0.5 & 0.1, 2.5 & 0.1, 5.0 & 0.1, 0.5 & 0, 2.5 & 0 and 5.0 & 0 mg/L, respectively, and one control were assayed. Highest frequency of green spot formation and plant regeneration (84% in Inqilab-91 and 52% in Pavon-76) was achieved at 0.5 & 0.1 mg/L of BAP and IAA, respectively. Higher concentrations of BAP in the media proved to be toxic and fatal for callus in some cases. Significant difference was observed among cultivars to callus *in vitro* and regenerate whole plants.

Introduction

The technology of growing cereal plants either from somatic or haploid cells has provided exciting new potential for plant improvement (Mohmand, 1989). Various explants have been tried to initiate regenerable cultures, among which immature embryos have been the most widely used in many cereals (Bhaskaran & Smith, 1990), but this approach is lengthy and resource intense. A more convenient source would be the mature caryopsis as it can prevent growing plants to the flowering stage in a greenhouse or a growth chamber (MacKinnon *et al.*, 1987).

Wheat cell and tissue culture research depends upon reliable callus cultures and plant regeneration procedures. Plant regeneration is one of the most critical steps of plant transformation (Keresa *et al.*, 2001). A number of workers have reported the regeneration of wheat plants from callus cultures derived from various plant parts (Yurkova *et al.*, 1981) but the frequency and duration of plant regeneration has usually been low (Conger, 1981). The successful application of plant biotechnologies to wheat improvement will largely depend on an ability to predictably regenerate plants from single cells (Carman *et al.*, 1988). Mature wheat embryos have a high frequency of callus induction and regeneration capacity (Ozgen *et al.*, 1998).

The aim of tissue culture studies has been to preserve and multiply the available germplasm using the least possible time and space (Quraishi *et al.*, 2000) and both the rate and occurrence of plant regeneration is cultivar specific (Schaeffer *et al.*, 1979;

¹Corresponding author (saad_uaar@yahoo.com)

²Agricultural Biotechnology Institute (ABI), NARC, Islamabad, Pakistan.

Sears & Deckard, 1982; Hanzel *et al.*, 1985; Arzani & Mirodjagh, 1999; Elwafa & Ismail 1999; Alok *et al.*, 1999), this study was conducted to establish a protocol for efficient plant regeneration from calli derived from mature wheat seed cultures of two genotypes (Inqilab-91 and Pavon-76). The mature seeds being available throughout the year can be used as an effective explant material in wheat callus induction and plant regeneration experiments. The protocol can be further used in developing transgenic wheat cultivars.

Materials and Methods

The present study was conducted in Agricultural Biotechnology Institute (ABI), NARC, Islamabad. Mature healthy seeds of two genotypes viz., Inqilab-91 and Pavon-76 of wheat (*Triticum aestivum* L., $2n = 6x = 42$) were washed with a commercial detergent under tap water. The seeds were then surface sterilized with a brief (30 sec) rinse with 95% ethanol followed by 20 min., vigorous wash with 30% Clorox® (1.5% sodium hypochlorite solution), plus one drop of Tween 80. The seeds were thoroughly washed six times with sterile distilled water in a laminar air-flow cabinet and were transferred to sterile Petri dishes having filter papers for drying. Linsmaier & Skoog's (1965) salts and vitamins, 2% (w/v) sucrose and 0.12% (w/v) Gelrite® were used as callus induction media supplemented with 3.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The media were adjusted to pH 5.75 and autoclaved for 17 min., at 121 °C.

Sterilized seeds of both varieties were inoculated on the gel-solidified, autoclaved media present in glass vials (18 mm in diameter and 150 mm in depth). 8 ± 1 mL medium was taken in each culture vessel and one seed was planted per culture vessel under sterile conditions. Cultures were transferred and maintained in environmentally controlled room under continuous illumination of 1500 lux emitted by general electric florescent tubes. Temperature was maintained at 25 ± 3 °C throughout the growth period. About 4-5 weeks were permitted for adequate induction and growth of calli for both genotypes. The calli when attained sufficient mass, were sub-cultured at least twice on the maintenance media. At the end of each culture passage, non-embryogenic (NE) callus, recognized on the basis of visual estimates, was dissected away from the embryogenic (E) callus and discarded. The subcultures were grown with same cultural conditions and media composition, except that 2,4-D level was reduced to 2 mg/L.

The plant regeneration ability of both genotypes was assessed using Murashige & Skoog's (1962) salts and vitamins, 3% (w/v) sucrose and 0.2% (w/v) Gelrite®. Seven different combinations of growth regulators, that is, BAP (6-benzylaminopurine) and IAA (Indole-3-acetic acid) were assayed to induce shoot and root differentiation and subsequent regeneration of plants. These combinations were 0 & 0.1, 0.5 & 0.1, 2.5 & 0.1, 5.0 & 0.1, 0.5 & 0, 2.5 & 0, 5.0 & 0 mg/L BAP and IAA, respectively, and one control.

Five weeks old calli carefully cleared of any surrounding NE callus were divided into small pieces (4-5 mm in diameter). These pieces were inoculated on the regeneration media present in the glass vials (18 mm in diameter and 150 mm in depth) and also in the glass jars (55 mm in diameter and 120 mm deep), separately, where each treatment comprised 6-7 cultures. Growth conditions and cultural procedures were similar to those for callus induction mentioned earlier. Calli bearing green spots and the total number of regenerates were counted after a time interval of 6-8 weeks. Number of plants regenerated was divided by the total number of calli planted to get regeneration percentage for each treatment. Each developing green shoot with an initiated root system was counted as one plant.

Results and Discussion

The callus that was compact, nodular and creamy white to light green in color bearing embryoid like structures and capable of regenerating plants, was recognized as E callus while the callus which was loose and watery in texture and dirty white in color was identified as NE callus. As there was no somatic embryogenesis observed in NE calli, these were found not to be regenerable. The somatic embryos may be recognized as the globular, somatic bipolar adventive structures derived from callus and capable of differentiating into whole plant.

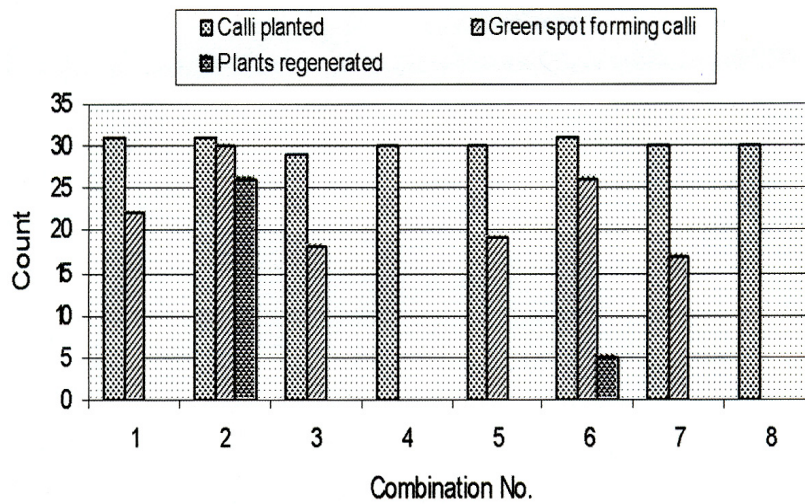


Fig. 1a. Number of calli planted, green spot forming calli and plants regenerated in Inqilab-91.

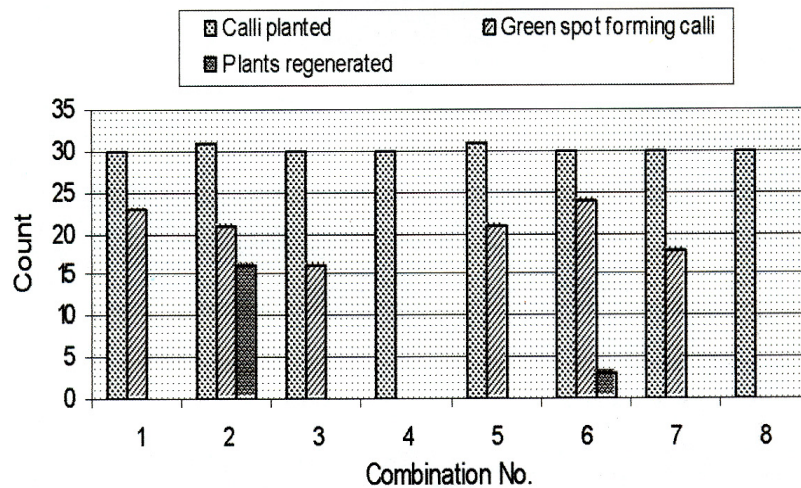


Fig. 1b. Number of calli planted, green spot forming calli and plants regenerated in Pavan-76.

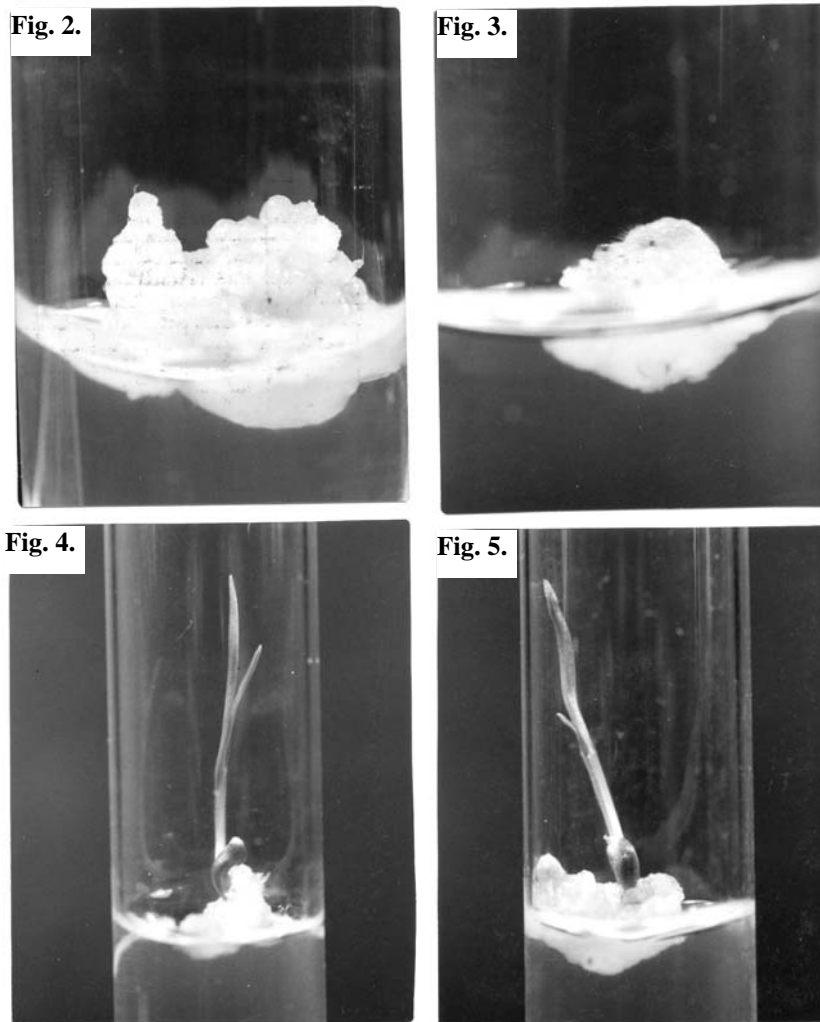


Fig. 2. Embryoids present on E callus.

Fig. 3. Green spot formation in callus on regeneration media.

Fig. 4. Shoot regeneration from E callus of Inqilab-91.

Fig. 5. Shoot regeneration from E callus of Pavon-76.

It was noticed that these embryoids or embryoid like structures (Fig. 2) present within the calli masses were responsible for green spot formation (Fig. 3) and, consequently plantlet development on regeneration media in both varieties. The highest number of green spot forming calli (Fig. 3) and the best regeneration rate was achieved on the media having 0.5 mg/L BAP and 0.1 mg/L IAA (Fig. 1a, b). When BAP level was increased to 2.5 mg/L, a few calli showed signs towards green spot formation but no root or shoot differentiation was seen. At elevated exogenous levels of BAP (5 mg/L), the calli turned brown in 10-15 days and, eventually, became dead. The concentrations of

IAA were used with slight differences among them, therefore, they didn't seem to have such a conspicuous effect like BAP. The percentage of plants regenerated at different BAP and IAA levels is shown in table 1.

In some cases, media supplemented with 0.5 mg/L BAP but no IAA allowed shoot and root differentiation. Although, the role of cytokinins (BAP) in conferring competence to regenerate in cereals is not very clear (Bhaskaran & Smith, 1990), but their low levels are known to promote shoot differentiation (Ignacimuthu, 1997) and shoot forming cultures have been able to produce viable plants by subsequent rooting (Bhaskaran & Smith, 1990). The role of IAA in whole plant regeneration seem not to be vital but it was found to increase whole plant regeneration by 68% and 42% in Inqilab-91 and Pavon-76, respectively, as compared to if BAP is present alone. Media supplemented with 0.1 mg/L IAA but without BAP, and control gave no signs towards plant regeneration. Several root primordia were observed to protrude out of the calli masses but no shoot or root differentiation was noticed.

Table 1. Plant regeneration in both genotypes under different concentrations of BAP & IAA.

Combination No.	Growth regulators (mg/L)		Regeneration (%)	
	BAP	IAA	Inqilab-91	Pavon-76
1	0	0.1	0	0
2	0.5	0.1	83.87	51.61
3	2.5	0.1	0	0
4	5.0	0.1	0	0
5	0	0	0	0
6	0.5	0	16.13	10
7	2.5	0	0	0
8	5.0	0	0	0

Regeneration potential differed significantly in both genotypes tested. The regeneration was 84% in Inqilab-91 while it remained 52% in Pavon-76. Green spot formation initiated 2 weeks after inoculation and shoot differentiation after 4 weeks, in a few calli, and lasted up to 8 weeks. In the initial phase of plant regeneration, small shoots emerged out of the somatic embryos in both genotypes (Figs. 4, 5) and after 2 weeks of shoot differentiation, root began to bulge out in the form of fine threads which later became prominent and distinguishable. These plantlets seem to be phenotypically normal and good representative of those germinated under normal field conditions.

Conditions optimal for plant regeneration in one cultivar fail to produce plants in another cultivar of the same species (Bhaskaran & Smith, 1990). This has been a nagging problem in cereal tissue culture, and may have contributed to the abundant literature on cereal tissue culture, as investigators try to optimize conditions for individual cultivars. In order to explore the actual potential for *in vitro* systems in wheat, large number of genotypes must be screened to identify those that allow E callus growth, and predictable and dependable plant regeneration so that they can be manipulated using modern genetic transformation techniques. Search of such genotypes and to optimize conditions for the development of high frequency plant regeneration systems is an important step towards application of tissue culture to plant breeding. Additionally, these genotypes could be a valuable source of material for selection studies, and somaclonal variations can be exploited in certain breeding programmes.

References

- Alok, V., S. Jain, S.L. Kathari, A. Varshney and S. Jain. 1999. Plant regeneration from mature embryos of 20 cultivars of wheat (*Triticum aestivum* L. and *T. durum* Desf.). *Cereal Res. Comm.*, 27(1-2): 163-170.
- Arzani, A. and S.S. Mirodjagh. 1999. Response of durum wheat cultivars to immature embryo culture, callus induction and *in vitro* salt stress. *Plant Cell, Tissue & Organ Culture*, 58(1): 67-72.
- Bhaskaran, S. and R.H. Smith. 1990. Regeneration in cereal tissue culture: A review. *Crop Sci.*, 30: 1328-1336.
- Carman, J.G., N.E. Jefferson and W.F. Campbell. 1988. Induction of embryogenic *Triticum aestivum* L. calli. I. Quantification of genotype and culture medium effects. *Plant Cell, Tissue & Organ Culture*, 12: 83-95.
- Conger, B.V. 1981. Agronomic Crops. In: *Principles and Practices of Cloning Agricultural Plants via In Vitro Techniques*. (Eds.): B.V. Conger, CRC Press, Boca Raton, Florida. pp. 165-216.
- Elwafa, A.A.A. and A.E.A. Ismail. 1999. Callus induction and plant regeneration from cultures of immature embryos of spring wheat. *Assiut. J. Agric. Sci.*, 30(3): 13-23.
- Hanzel, J.J., J.P. Miller, M.A. Brinkman and E. Fendos. 1985. Genotype and media effects on callus formation and regeneration in barley. *Crop Sci.*, 25(1): 27-31.
- Ignacimuthu, I. 1997. *Plant Biotechnology*. Oxford & IBH Pub. Co., New Delhi, India. pp. 201-203.
- Keresa, S., M. Baric, H. Sarcevic, S. Marchetti and G. Dresner. 2001. Callus induction and plant regeneration from immature and mature embryos of winter wheat (*Triticum aestivum* L.) genotypes. *Plant Breeding: Sustaining the future*. Abstracts of the XVIth EUCARPIA Congress, Edinburgh, Scotland.
- Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*, 18: 100-127.
- MacKinnon, C., G. Gunderson and M.W. Nabors. 1987. High efficiency plant regeneration by somatic embryogenesis from callus of mature embryo explants of bread wheat (*Triticum aestivum*) and grain sorghum (*Sorghum bicolor*). *In Vitro Cell. Dev. Biol.*, 23(3): 443-448.
- Mohmand, A.S. 1989. *Stress selection, osmoregulation, somaclonal variation, and long term plant regeneration in wheat*. Ph. D. dissertation, Deptt. of Botany, Colorado State University, Fort Collins, USA.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15: 473-497.
- Ozgen, M., M. Turet, S. Altinok and C. Sancak. 1998. Efficient callus induction and plant regeneration from mature embryo cultures of winter wheat (*Triticum aestivum* L.) genotypes. *Plant Cell Reports*, 18(3-4): 331-335.
- Quraishi, A., F. Tahir and H. Rashid. 2000. Effect of NaCl on callogenesis of *Triticum aestivum* L. *Pakistan J. Agric. Res.*, 16(1): 28-30.
- Schaeffer, G.W., P.S. Baenziger and J. Worley. 1979. Haploid plant development from anther and *in vitro* embryo culture of wheat. *Crop Sci.*, 19: 697-702.
- Sears, G.R. and E.L. Deckard. 1982. Tissue culture variability in wheat: Callus induction and plant regeneration. *Crop Sci.*, 22: 446-450.
- Yurkova, G.N., B.A. Levenko and D.V. Novozhilov. 1981. Induction of plant regeneration in wheat tissue culture. *Biochem. Physiol. Pflanzen.*, 176: 236-243.

(Received for publication 18 August 2003)