SOMATIC EMBRYOGENESIS AND LONG TERM HIGH PLANT REGENERATION FROM BARLEY (*HORDEUM VULGARE* L.) USING PICLORAM

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Summary

Long term high plant regeneration was obtained from embryogenic callus induced from immature embryo explants of *Hordeum vulgare* cv RD-57 cultured on MSB medium supplemented with Picloram (2 and 4mg l^{-1}). In the subsequent subcultures different types of calli were recognised which could be distinguished from each other on the basis of their morphology, growth and plant regeneration ability. Best growth and maintenance of embryogenic state of callus was achieved on MSB medium supplemented with Picloram (2 mgl^{-1}). For plant regeneration, embryogenic callus was transferred to hormone free MSB medium. Regenerated plantlets were rooted either on hormone free MSB medium or MSB medium supplemented with IAA (0.5 mgl^{-1}). The regenerated plants were transferred to field conditions where they matured and set seeds. Histological studies of embryogenic callus revealed all the stages of typical somatic embryogenesis. In addition, atypical somatic embryos and secondary somatic embryogenesis was also observed and occasionally *de novo* shoot morphogenesis was seen in the same cultures.

Index word : Somatic embryogenesis, plant regeneration, immature embryo, barley.

Introduction

During the last decade an impressive progress has been made on plant regeneration from callus, cell suspension and protoplast cultures of cereals (Vasil 1994, Kothari and Chandra 1995). But one major problem of cereal tissue culture has been the gradual loss of embryogenic capacity and regeneration potential during subcultures (Bregitzer 1991, Kachhwaha and Kothari 1994a). It is therefore, desirable to maintain and proliferate competent totipotent cell cultures for several passages and also to elucidate the pattern of growth and differentiation. Detailed histology and ontogeny of somatic embryogenesis in all major cereals and grasses has been reported. Examples : Wheat (Ozias-Akins and Vasil 1982, He *et al.* 1990), Rice (Jones and Rost 1989), Maize (McCain and Hodges 1986), *Pennisetum* (Botti and Vasil 1983, Vasil and Vasil 1985), *Sorghum* (Dunstan *et al.* 1978, 1979), *Panicum* (Lu and Vasil 1985, Kothari *et al.* 1994a), Eleusine (Wakizuka and Yamaguchi 1987, Sivadas *et al.* 1992) and Saccharum (Ho and Vasil 1983). Plant regeneration from tissue culture of barley has been reported (Kothari and Chandra 1988, Bhaskaran and Smith 1990, Kachhwaha and Kothari, 1996), but there have been conflicting reports on the mode of plant regeneration. Some workers report it to be organogenesis (Cheng and Smith 1975, Jelaska *et al.* 1984, Seguin-Swartz *et al.* 1984, Luppoto 1984, Bhattacharya 1991), while others find it to be somatic embryogenesis (Kott and Kasha 1984, Goldstein and Kronstad 1986). However, no detailed histological studies have been

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carried out to understand the exact mode of plant regeneration in *H. vulgare*. Also it has been noted that in most of these reports 2,4-D was the only plant growth regulator used for evoking morphogenetic response. In the present study, Picloram proved better than 2,4-D for induction and long term maintenance of regenerable callus from immature embryo explants of *H. vulgare*. Histological details of pathways of regeneration in barley have also been described in this paper.

Materials and Methods

Seeds of *Hordeum vulgare* L. cv RD-57 were obtained from Agriculture Research Station, Durgapura, Jaipur, India. The immature caryopses were taken from plants grown under field conditions and were surface sterilized in 0.1% $HgCl_2$ for 3-4 minutes and rinsed in sterile distilled water thrice. The immature embryo (1-1.5mm long) were dissected out and cultured on MSB medium, containing Murashige and Skoog mineral salts (Murashige and Skoog 1962) and Gamborg *et al.*, vitamins (Gamborg *et al.* 1968) with 3% sucrose, 0.8% agar (Qualigens, India) and pH 5.8. The medium was supplemented with Picloram or 2,4-D (2 and 4 mgl^{-1}). Fifty immature embryos were taken in ten replicates for each treatment. Five embryos were placed in a flask (Erlenmeyer, 100 ml) containing 40ml of medium. The cultures were incubated at $26 \pm 2^{o}C$ in dark. Callus induced was subcultured every four weeks and maintained on medium with 2 mgl^{-1} of Pic or 2mg/l 2,4-D and was kept under 16 h photoperiod (1400 lux) and 26±2°C temperature. For plant regeneration approximately 1 gm of embryogenic callus was transferred to hormone free MSB medium. Callus cultures containing small plantlets were transferred either to hormone-free MSB medium or MSB medium supplemented with IAA (0.5 mgl^{-1}) for root induction.

Plantlets with well developed root and shoot systems were taken out from culture vessels. The roots were thoroughly washed under running tap water to remove agar. After washing, the plantlets were transferred to 10 cm diameter earthen pots containing normal garden soil. The pots were kept under field conditions. No hardening pretreatment of any type was given to the plants.

For histological studies, different embryogenic calli were fixed in FAA. The fixed material was then dehydrated through a graded tertiary-butyl alcohol series, infiltrated and embedded in paraffin wax. Serial sections (10-11 μm) were cut with the help of a rotary microtome (Weswox), stained in safranin-fast green and mounted in DPX (Johansen 1940).

Results

Callus initiation from immature embryo explants was observed within the first week of culture on MSB medium supplemented with Pic (2 and 4 mgl^{-1}). The callus induced was soft, white and friable. The callus became slightly organised during late periods of culture (4th week). The callus induced was subcultured on MSB medium supplemented with 2 mgl^{-1} of Pic after 30 days and was kept under 16 h photoperiod. The primary callus induced on 2 and 4 mgl^{-1} of Pic differed in terms of fresh weight and could be broadly classified into four types on the basis of their morphology, growth rate and regeneration potential (Fig. 1A).

These callus types were often closely associated and it was difficult to separate them from one another. These calli were:

Type A Callus : White, convoluted and relatively friable with several green spots which later on sprouts into many leafy structures. This callus had moderate growth rate and regeneration potential (Fig. 1B).
Type B callus : Creamish-brown, soft and mucilaginous callus, typically non morphogenic in appearance but later on gave rise to many plantlets. This type of callus had good growth rate but lost its regeneration potential within five sub-cultures (Fig. 1C).
Type C callus : White and very fast growing soft non embryogenic callus. This callus was incapable of regeneration but could be easily maintained and proliferated in subsequent subcultures (Fig. 1D).
Type D callus : White, compact, shiny, nodular, morphogenetically competent callus. This type of callus has slow growth but good regeneration potential (Fig. 1E).

Table 1 *In vitro* response of immature embryos of *H. vulgare* cv RD-57 on MSB Medium.

Growth regulators (mgl^{-1})	Number of embryos plated	Number of embryos forming embryo-genic callus	Mean Fresh weight of embryogenic callus after first subculture ± Standard error (gm) *	Mean Number (± Standard error) of plantlets regenerated from 1 gm of embryogenic callus during subcultures		
				2nd subculture	10th subculture	15th subculture
Pic.						
(2.0)	50	50	1.26±0.15	23.2±2.6	16.4±1.3	6.86±1.3
(4.0)	50	50	2.82±0.62	33±4.1	4.6±0.6	-
2,4-D						
(2.0)	50	50	0.73±0.12	6.4±1.0	-	-
(4.0)	50	50	1.21±0.13	12±1.2	-	-

* Callus formed on Picloram or 2,4-D containing media in the primary culture was subcultured on lower levels of Picloram or 2,4-D (2 mgl^{-1}) in the subsequent passages.

** Regeneration medium contained plain MSB medium without any growth regulator.

The embryogenic callus (Type A and D) was maintained for more than one year by repeatedly subculturing it on MSB medium supplemented with 2 mgl^{-1} Pic. On this maintenance medium, cultures produced large amounts of embryogenic callus. Well developed somatic embryos germinated forming leafy appendages while less differentiated structures recallused. Differentiated embryogenic callus was transferred to a hormone free medium for plant regeneration (Fig. 1F). The efficiency of callusing and plant regeneration from the callus induced and subcultured on Pic supplemented media was higher in comparison to the cultures induced and maintained on the same basal medium but supplemented with 2,4-D. Callus retained its embryogenic potential on Pic supplemented media for 18 subsultures while on the 2,4-D supplemented media, embryogenic potential of the callus was lost after 6 subcultures. The amount of callus formed and number of regenerated plants from callus induced and maintained on Pic supplemented media was

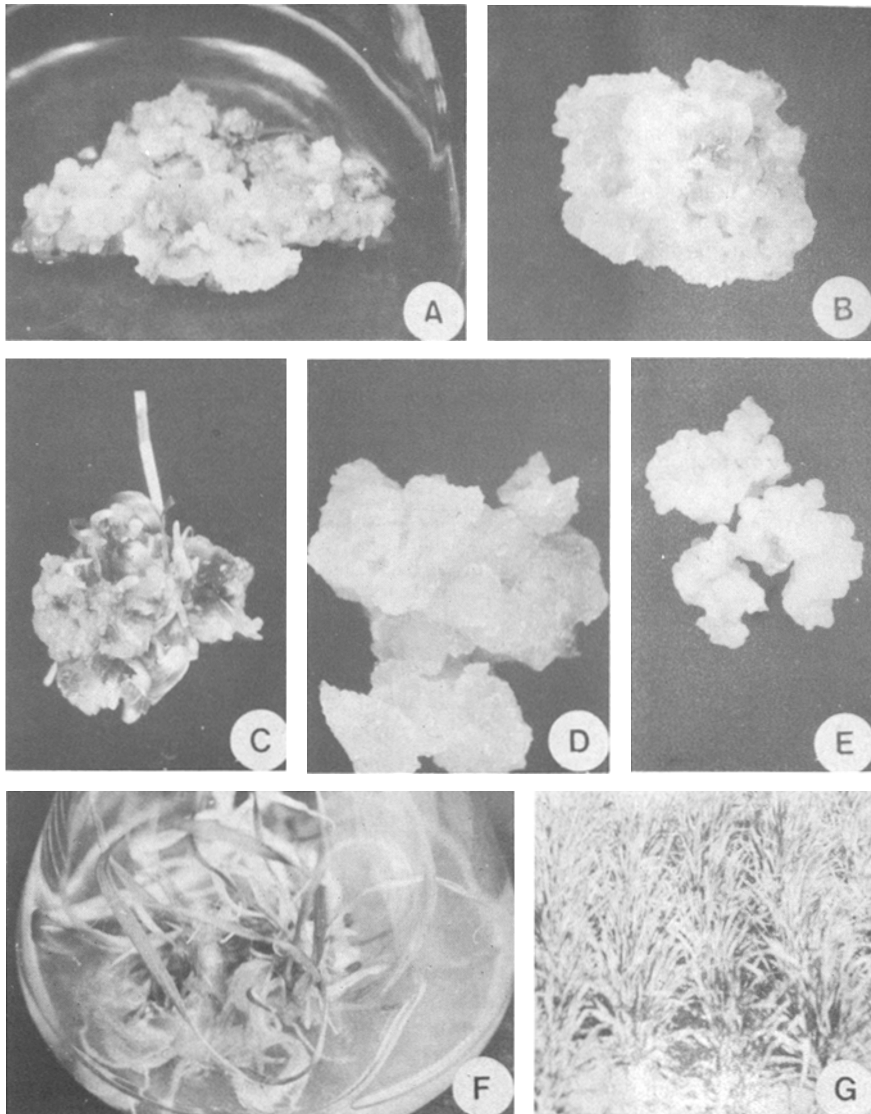

Fig. 1. A. Embryogenic callus after first subculture on Picloram (2.0 mgl^{-1}) supplemented MSB medium; B. Type A callus; C. Type B callus; D. Type C callus; E. Type D callus; F. Plant regeneration from embryogenic callus on plain MSB medium.; G. R_1 seed progeny of *in vitro* plants in the field.

higher than callus induced and maintained on 2,4-D containing media (Table 1). The results indicate that Pic is superior to 2,4-D for embryogenic callus initiation, long term maintenance and growth. As the embryogenic callus became old (11 month onwards) albino plants as well as green plants with distorted appearance were seen to be formed and this tendency of the cultures increased and became pronounced after 12 months of callus induction. For the induction of root system small plantlets were transferred either to plain MSB or MSB medium supplemented with IAA (0.5 mgl^{-1}). The regenerated plantlets were transferred to pots containing garden soil under field conditions without any hardening. All the plantlets survived very well and set seeds. Seed progeny of R_1 plants has been raised to study the somaclonal variations (data not shown, Fig. 1G).

Histological studies of all the three different type of embryogenic calli (Type A,B and D) was carried out separately and the mode of plant regeneration was found to be similar in all cases. It was observed that due to continued meristematic activity of some cells in the callus, lobed embryogenic callus tissue was formed. Continued divisions and organisation in this tissue gave rise to rounded or elongated proembryoids, with distinct epidermis (Fig. 2A). The development of the embryoids was not synchronous and various stages of development could be traced adjacent to each other. Proembryoids and embryoids were attached to the callus surface by a small suspensor (Fig. 2A). However, in many cases the suspensor was indistinguishable or present as a broad multicellular structure. Further differentiation led to the formation of a lateral cleft in the terminal parts of the embryoids (Fig. 2B). Increased growth and organisation led to the formation of distinct bipolar embryoids with a scutellum, a coleoptile and a coleorhiza (Fig. 2C). In several cases large peripheral starch containing cells of the scutellum proliferated to give rise to secondary embryoids. Besides the formation of typical embryoids, atypical mode of embryogenesis was also found to be of common occurrence. The scutellar part of many embryoids became green and produced few leaves (Fig. 2D). In many embryoids it was observed that due to increased meristematic activity in the region of shoot apex, several shoot meristems were formed and a coleoptile was also present. These multiple shoots were seen to be connected to only one root meristem formed at the opposite end of the multiple shoots (Fig. 2E). Occasional *de novo* shoot morphogenesis was also observed containing an apical meristem and two leaf primordia on the surface of callus. These shoot primordia were broadly attached to the parent tissue and were not associated with any root meristems (Fig. 2F). The embryogenic state of the callus was primarily maintained during the subcultures by the formation of *de novo* somatic embryos as well as the formation of secondary somatic embryos.

Discussion

In cereals, the auxin 2,4-D is most commonly used for the induction of somatic embryogenesis in *in vitro* cultures (Sharma *et al.*, 1989, Bhaskaran and Smith 1990, Kothari *et al.*, 1994b, Vasil 1994, Oinam and Kothari, 1995, Sharma *et al.*, 1995). An interesting finding of this study is the successful use of Pic. as an auxin source for the production of embryogenic callus which is capable of long-term high plant regeneration in barley. Ho and Vasil (1983) found Pic (0.1-6.0 mgl^{-1}) not effective for the induction of embryogenic callus in *Saccharum*, while Eapen and George (1989) reported somatic embryogenesis induced

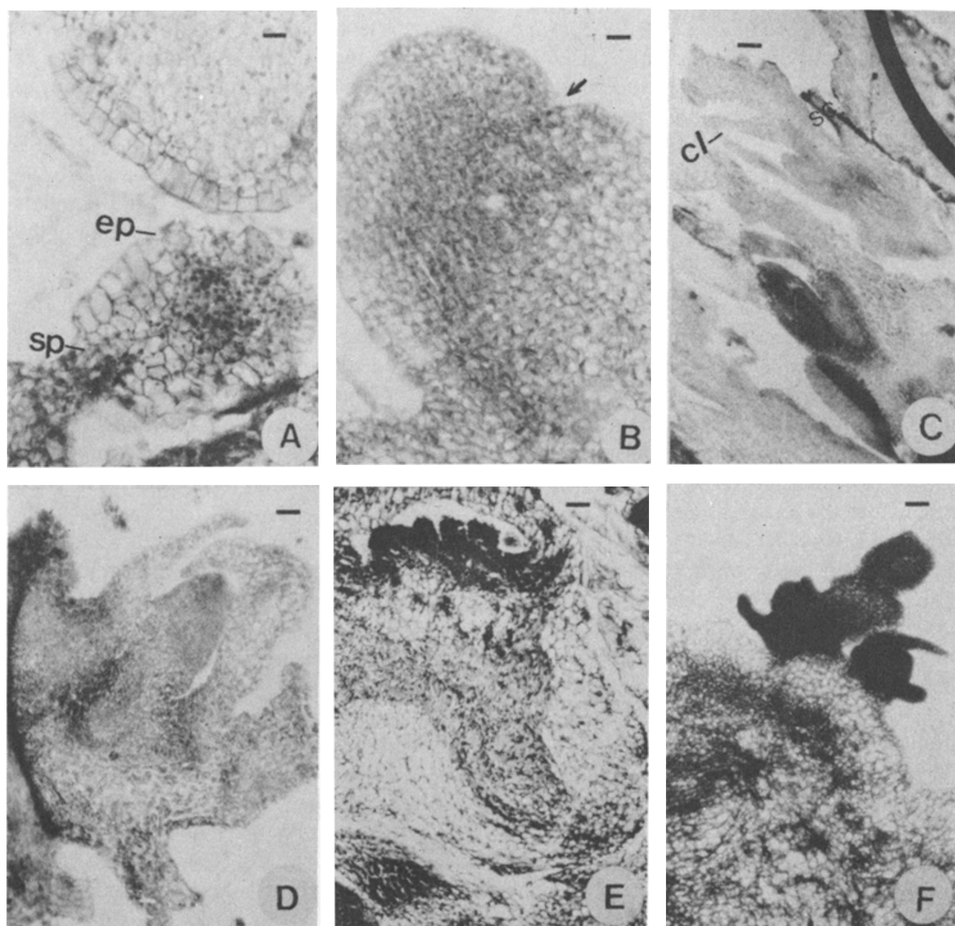

Fig. 2. A. Longitudinal section of somatic embryo with well defined epidermis (ep) and suspensor (sp). (Bar = 50 µm); B. Longitudinal section of an embryoid soon after the formation of the lateral notch (arrow head). (Bar = 50 µm); C. Bipolar somatic embryo with distinct coleoptile (cl), scutellum (sc), shoot, and root pole. (Bar = 125 µm); D. Atypical embryoids germinating precociously with several leafy structures. (Bar = 50 µm); E. Atypical embryoid with broad shoot apex giving rise to multiple shoots. (Bar = 50 µm); F. Shoot buds developed on the surface of callus tissue. (Bar = 125 µm)

from shoot apices of finger millet on MS basal medium supplemented with Pic (4 mgl^{-1}) and kinetin (0.5 mgl^{-1}). In *H. vulgare*, Luhrs and Lorz (1987) though reported Pic (2mgl^{-1}) suitable for embryogenic callus induction but no details were given. For barley cell suspensions Luhrs and Lorz (1988) reported Pic (4-8 mgl^{-1}) to cause necrosis of the incubated cells while Jahne *et al.* (1991) observed that medium supplemented with Pic (2mgl^{-1}) resulted in the production of soft non embryogenic callus. In barley tissue culture one of the common problems faced is the maintenance of the embryogenic callus. Callus induced on 2,4-D was often found to be difficult to maintain in an embryogenic state for a long period (Bregitzer 1991, Kachhwaha and Kothari 1994b, Luhrs and Lorz 1987). But the use of Pic in the present study provides good callus growth in maintenance cultures and results in high plant regeneration also.

In the present study we recognized different types of calli which were morphologically distinct, our observations are consistent with that of Orton (1979) who characterized five morphologically distinct categories of callus in barley exhibiting a range of regeneration potential. Many other workers have also recognized different type of callus formation in *H. vulgare* (Seguin-Swartz *et al.* 1984, Lupotto 1984, Goldstein and Kronstad 1986, Bregitzer 1991).

Consistent with the observations made in other cereals by Vasil and Vasil (1982), Botti and Vasil (1983),Ho and Vasil (1983), Jones and Rost (1989) in the present study we traced all the early to late stages of somatic embryogenesis in *H. vulgare*. It has been reported by several workers that barley embryoids are less typical of grass embryos in morphology than are embryoids of other grass species (Kott and Kasha 1984, Thomas and Scott 1985, Weighel and Hughes 1985, Kachhwaha and Kothari 1994b). We also hold the same view and our studies also indicate that the majority of embryoids that produced plantlets were atypical. In agreement with the observations made by Weighel and Hughes (1985) in *H. vulgare*, Vasil *et al.* (1985) in maize and He *et al.* (1990) in wheat we also observed *de novo* shoot morphogenesis in the same cultures along with embryogenesis in barley. Thus, though somatic embryogenesis is the predominant pathway of morphogenesis in barley but *de novo* shoot morphogenesis also occur either in close association or independently of somatic embryos (Kachhwaha and Kothari 1994 a,b).

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