INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2003/06–1–156–159
http://www.ijab.org

Effect of 2,4-dichlorophenoxyacetic Acid on Callus Induction from Mature Wheat (*Triticum aestivum* L.) Seeds

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ABSTRACT

Mature healthy seeds of two genotypes of wheat viz. Inqilab-91 and Pavon-76, were inoculated on the gel-solidified LS media supplemented with different concentrations of 2,4-D. Callus induction frequency differed significantly in both genotypes tested and remained 48 and 100% in Inqilab-91 and Pavon-76, respectively. Higher levels of 2,4-D were found to inhibit callus proliferation while lower concentrations allowed morphogenesis to occur. It was found that 3.5 mg/L 2,4-D was the most appropriate concentration in LS media for adequate callus induction and proliferation, and 2 mg/L for subsequent subcultures from mature wheat seeds. Good embryoid production was apparent in third and fourth weeks of callus subculture. Average fresh weights of the calli for Inqilab-91 and Pavon-76 were 0.37 and 0.36 g per explant source, respectively. Calli fresh weights were found to be significantly and positively correlated with dry weights.

Key Words: Wheat; Callus; Mature seeds; 2,4-dichlorophenoxyacetic acid

INTRODUCTION

The yield and quality of wheat have been gradually improved during the past several decades by traditional breeding methods. However, these methods have some limitations such as long time required and rather limited gene pool available for wheat breeders. For this purpose, a group of activities was focused on *in vitro* culture and regeneration as a tool of cereal breeding in the recent years. Further, it is also well documented that the genetic engineering of cereals currently depends on the use of tissue culture and plant regeneration (Mendoza & Kaeppler, 2002). In wheat, immature embryos have been the most widely used explants to initiate callus cultures (Bhaskaran & Smith, 1990; Felfoldi & Purnhauser, 1992; Ozgen *et al*., 1996; Hou *et al*., 1997; Arzani & Mirodjagh, 1999) but they are inconvenient because of their temporal availability (MacKinnon *et al*., 1987; Mendoza & Kaeppler, 2002). Mature seeds are readily available throughout the year, hence can be used effectively to initiate callus cultures in wheat. Ozgen *et al*. (1998) reported high frequency of callus induction through mature embryos, however, they found it low while working with seven durum wheat genotypes in one of their previous studies, where mature embryos were used as explant source (Ozgen *et al*., 1996).

The callus is a rapidly proliferating undifferentiated mass of cells, which can be obtained by culturing explants on nutrient media containing specific growth hormones (Naqvi *et al*., 2002). 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin and is the most commonly used growth regulator in cereal tissue culture (He *et al*., 1986; Mikami & Kinoshita, 1988; Ozawa & Komamine, 1989; Bregitzer *et al*., 1989; Naqvi *et al*., 2002). In wheat, 2,4-D alone or in combination with cytokinins has been used for callus initiation (Mathias *et al*., 1986; Lazer *et al*., 1988). Different genotypes are reported to respond differently to callus *in vitro* under different 2,4-D concentrations (Elwafa & Ismail, 1999). The focus of this study was to optimize cultural conditions and to find suitable 2,4-D levels for efficient callus induction and growth in two wheat genotypes, Inqilab-91 and Pavon-76. Starting material being available throughout the year can be efficiently used as initial explant for induction of calli and subsequent growth. The protocol was further used to regenerate whole plants.

MATERIALS AND METHODS

Seed source. This study was conducted in Agricultural Biotechnology Institute (ABI), NARC, Islamabad. Mature seeds of two genotypes, Inqilab-91 and Pavon-76 of wheat (*Triticum aestivum* L.) were kindly provided by the Wheat Programme, NARC, Islamabad.

Sterilization procedure. Selected healthy seeds of both genotypes were washed with commercial detergent (Zip) under tap water following surface sterilization with a brief (30 sec) rinse with 95% ethanol. The seeds were then treated for 20 min with 30% Clorox® (commercial bleach of 5.25% sodium hypochlorite solution) plus one drop of tween-80 per 200 seeds in a sterilized 100 mL Erlenmeyer flask. The seeds were taken to the laminar air flow cabinet. They were thoroughly washed six times with sterilized distilled water and were shifted separately to sterilized petri plates having filter papers for drying.

Callus induction media. Linsmaier and Skoog's LS (1965) salts and vitamins, 2% (w/v) sucrose, 0.12% (w/v) Gelrite® were used as callus induction medium. The media were

supplemented with various concentrations (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L) of 2,4-D. The pH was adjusted to 5.75 prior to autoclaving. About 8 ± 1 mL medium was taken in each test tube and the test tubes were plugged with cotton. Media were autoclaved at 17 psi for 20 min and allowed to cool down and solidify at room temperature.

Inoculation. Seeds of both varieties were inoculated on the gel-solidified LS media present in the test tubes (18 mm in diameter and 150 mm in depth) in a laminar air-flow cabinet under sterilized conditions. One seed per test tube was planted vertically, keeping the scutellum side up in such a way that one-third portion of the seed remained above the surface of the media.

Growth conditions. Cultures were transferred and incubated in an environmentally controlled room. They were positioned vertically, 10 inches away from continuous light of approximately 1500 lux provided by general electric florescent tubes. Temperature was maintained at 25 ± 3 ºC throughout the growth period.

Culture period. Four to five weeks were permitted for adequate induction and growth of calli in both genotypes. About one week was spent in data recording and transferring.

Growth measurement. Callus induction frequency for both genotypes was assessed 10 days after inoculation. Callus proliferation rate and callus quality was recorded 4-5 weeks after inoculation in both varieties for all treatments. Direct weighing of calli from each culture vessel proved to be a laborious and time-consuming task. The calli masses were then visually scored as good, average and poor. Additionally, their fresh and dry weights were taken.

Fresh and dry weights. Ten calli (35 days old) for each genotype were selected randomly out of top 50% calli. The seed and other unwanted materials were removed. The calli were weighed individually on an electrical balance and their fresh weights were taken in grams. The calli were then oven dried at 65 ºC for 18 h and their dry weights were calculated.

Callus maintenance. Cultures with appreciable callus induction and growth were shifted to the laminar air-flow cabinet. The calli were collected in sterile petri plates and were carefully cleared of any unwanted material. The non-embryogenic (NE) callus was separated by visual estimates from the embryogenic callus (E callus) as described by MacKinnon *et al.* (1987) and discarded. Clumps of callus were divided into 3 to 4 mm sized pieces and were transferred to the maintenance medium similar to that of callus induction medium except that 2,4-D level was reduced to 2 mg/L. This level promotes callus proliferation as compared to its higher concentrations, but inhibits organogenesis in the E callus. The cultures were maintained for 4-5 weeks on the maintenance medium following subsequent subcultures until they were transferred to the regeneration medium.

RESULTS AND DISCUSSION

Callus induction began after five days in culture in most of the explants and lasted up to four weeks rapidly, following less frequent proliferation during subsequent time periods. Callus induction and shooting progressed simultaneously, but in the third week of shoot development, further shoot growth terminated while calli kept on increasing in their masses. It was found that higher concentrations of 2,4-D inhibit callus proliferation and lower concentrations allowed morphogenesis to occur (Table I), which affect both callus quantity and quality (Fig. 5). Hence, medium supplemented with 3.5 mg/L 2,4-D was found optimum for callus induction from physiologically mature wheat seeds. Optimal callus induction could be obtained through manipulating 2,4-D concentrations and the duration of its presence in the induction medium (Zheng & Konzak, 1999) as different genotypes respond differently to varying 2,4-D levels (Arzani & Mirodjagh, 1999).

Table I. Callus induction rate and shoot development (shooting) from seeds of both varieties on LS media at different 2,4-D levels

2,4-D Concentration (mg/L)	Callus induction	Shooting
1.5	+	++++
2.0	+	+++
2.5	++	+++
3.0	+++	++
3.5	++++	++
4.0	+++	+

++++ Vigorous +++ Good ++ Average + Poor

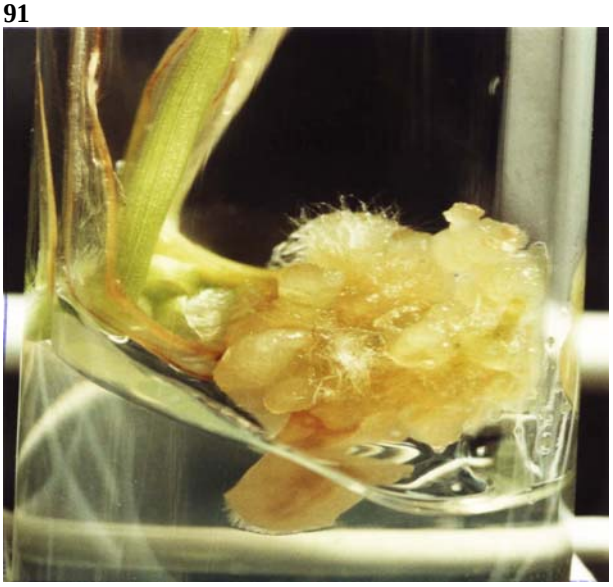
Table II. Fresh and dry weight comparison of calli of Inqilab-91 and Pavon-76

No.	Fresh weights (g)		Dry weights (g)	
	Inqilab-91	Pavon-76	Inqilab-91	Pavon-76
1	0.31	0.32	0.038	0.048
2	0.32	0.33	0.044	0.053
3	0.34	0.35	0.045	0.055
4	0.35	0.36	0.047	0.055
5	0.36	0.36	0.047	0.057
6	0.36	0.38	0.048	0.059
7	0.38	0.38	0.049	0.060
8	0.40	0.39	0.049	0.061
9	0.41	0.42	0.051	0.064
10	0.41	0.45	0.052	0.065
Average	0.364	0.374	0.047	0.057

Table III. Varietal response to callus induction frequency and callus quality at 3.5 mg/L 2,4-D

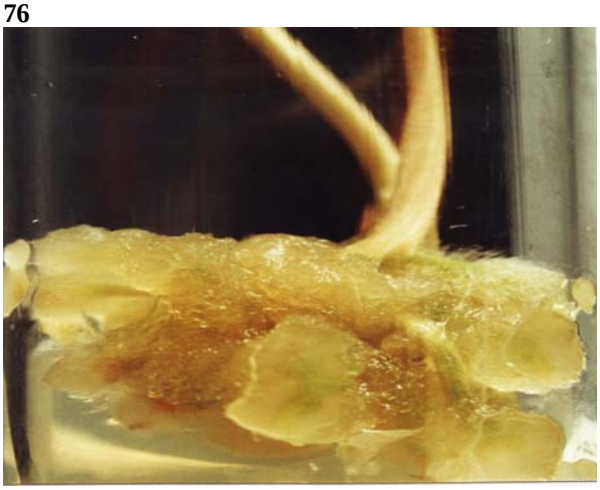
Varieties	Callus induction frequency (%)	Callus quality (%)		
		Good	Average	Poor
Inqilab-91	48	33	20	47
Pavon-76	100	31	21	48

Fig. 1. Callus induction from mature seed of Inqilab-91



Callus induction frequency differed significantly in both genotypes tested but less difference was observed,

Fig. 2. Callus induction from mature seed of Pavon-76



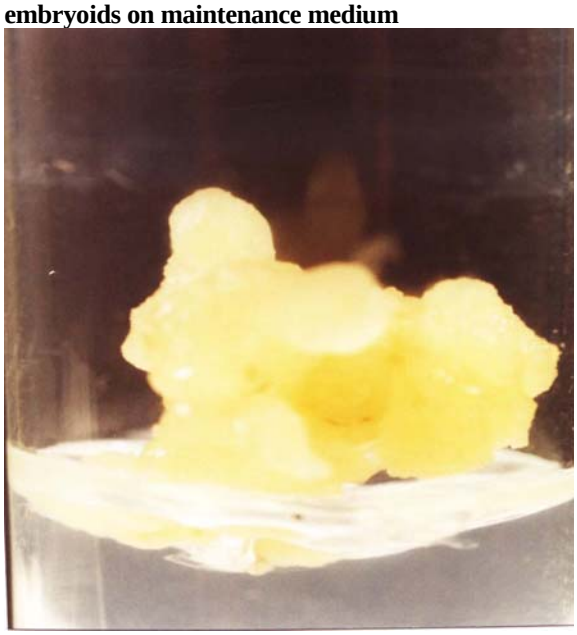
comparatively, regarding callus quality. Callus induction frequency was 48% in Inqilab-91 while it remained 100% in Pavon-76 (Table III). Ozgen *et al*. (2001) found cytoplasm to be positively affecting callus induction and culture efficiency in wheat while Gonzalez *et al*. (2001) reported strong dependence of callus induction on genotype and induction media used.

In both genotypes, two distinct callus types were observed. A growing unorganized collection of embryoid or embryoid like structures proliferating from the scutellum of the seed was recognized as embryogenic callus (E callus). This callus was compact, noduled and off-white to light green in color and was capable of proliferating globular embryoids or embryoid like structures in subsequent sub-

Fig. 3. E callus bearing globular embryoids on maintenance medium

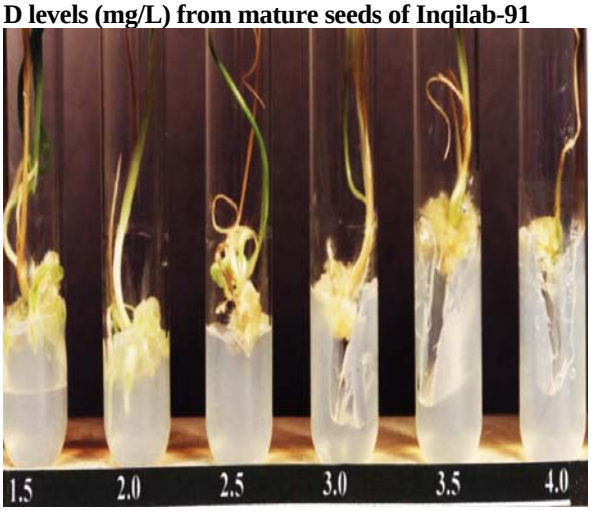
cultures (Fig. 3). Callus that was dirty white, brownish or grayish in color, watery and loose in texture was identified as non-embryogenic (NE) callus. Same observations are also reported by MacKinnon *et al*. (1988). No embryoids or embryoid like structures were present on such type of calli (Fig. 4).

Fig. 4. NE callus loose and watery in texture with no embryoids on maintenance medium



Fresh and dry weights of whole calli were taken after 35 days of callus induction for both varieties (Table II). Average fresh weight for Pavon-76 was 0.37 g and for Inqilab-91 it was 0.36 g per explant source. Fresh weights

Fig. 5. Callus induction and shooting at different 2,4-D levels (mg/L) from mature seeds of Inqilab-91



were found to be significantly and highly correlated with dry weights in Inqilab-91 (r = 0.92) and Pavon-76 (r = 0.96). Similarly, the average dry weights were determined as 0.047 g and 0.058 g for Inqilab-91 and Pavon-76, respectively (Table II). Ozias-Akins and Vasil (1982) found increased concentration of 2,4-D in the media to be negatively correlated with increase in fresh weight and total cell number.

E callus seemed not to grow as fast as NE callus when sub-cultured on reduced 2,4-D concentration of 2 mg/L. It seemed essential to separate NE callus to maintain embryogenic calli in subsequent sub-cultures. Good embryoid production was seen during third and fourth weeks of sub-culture. 2 mg/L 2,4-D was chosen as the optimum level for callus proliferation and growth in the sub-culture media. According to Ozias-Akins and Vasil (1982) increased level of 2,4-D beyond 2 mg/L inhibit cell division.

Optimizing cultural conditions, development of efficient protocols for *in vitro* cultures and screening of wheat genotypes for tissue culture response may play an important role in crop improvement by shortening the time needed to release new varieties or introducing new variability in the existing material. Impact of such technologies is tremendous and hence demands its integration with conventional plant breeding methods.

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(Received 10 October 2003; Accepted 23 November 2003)