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Full Length Research Paper

Effect of age on somatic embryogenesis from immature zygotic embryos of 5 Turkish triticale genotypes

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Triticale is an important cereal crop grown throughout the world. The study reports somatic embryogenesis from immature zygotic embryos of 5 Turkish triticale genotypes. The explants were initially cultured on MS medium supplemented with 2 mg dm^{-3} 2,4-D, 500 mg dm^{-3} glutamine, 100 mg dm^{-3} casein hydrolysate, 2% sucrose and 7% agar for two weeks in the dark. The temperature was maintained at 24±2ºC. Thereafter, the developing embryogenic calli were transferred to MS medium without 2,4-D to achieve embryogenesis under light intensity of 30 000 lux in 16 h light 8 h dark photoperiod at 24±2ºC for 2 weeks. The developing somatic embryos were then transferred to MS medium to mature them and obtain plantlets. The highest number of 9.63 somatic embryos per explant were recorded for genotype LAD388 from 14 - 16 days old explants. The experiment clearly established that 14 - 16 days old explants were superior compared to 17 - 19 days old explants in terms of number of somatic embryos per explant. The matured plantlets were acclimatised in the greenhouse on organic matter rich soil mix contained in pots.

Key words: Triticale, immature zygotic embryos, somatic embryogenesis, age.

INTRODUCTION

Triticale (x *Triticosecale* Wittmack) is a synthetic amphiploid cereal that grows on about 3 million hectares in the world (FAO, 2007). Depending on the cultivar, triticale can more or less resemble either of its parents. It is grown mostly for forage or animal feed although some triticale-based foods can be purchased at health food stores or are found in some breakfast cereals (Stallknech et al., 1996). The responses of cereals including rice and maize to tissue culture have been extensively studied over the years. Cereals such as wheat, barley, oat, rye and triticale are relatively recalcit- rant. Compared to other cereals, there are relatively few reports on *in vitro* tissue culture of triticale from mature or immature zygotic embryos (Nakamura and Keller, 1982; Eapen and Rao, 1985; Stolarz and Lörz, 1991; Stolarz, 1991; Immonen, 1992; Bohorova et al., 2001; Birsin and Ozgen 2004; Ganeshan et al., 2006). Successful microspore culture of triticale has been achieved by Immonen and Robinson (2000), Oleszczuk et al. (2004), Eudes and Amundsen (2005), Lantos et al. (2005) and Pauk et al. (2000). Similarly, microspore culture of triticale x wheat and triticale x triticale hybrids by Pratap et al. (2006) has also been reported. Relatively few tissue culture reports are available on mature or immature zygotic embryos of triticale when compared to other cereals.

It has therefore been necessary to optimize conditions for regeneration prior to attempting genetic transformation and selection of favorable somaclonal variant strains. The need for the development of highly efficient regeneration/transformation systems in triticale has become even more critical to keep pace with concomitant advances in functional genomics.

The relationship between the morphogenic potential

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Abbreviations: MS medium, Murashige and Skoog medium; 2,4-D, 2, 4- dichlorophenoxyacetic acid.

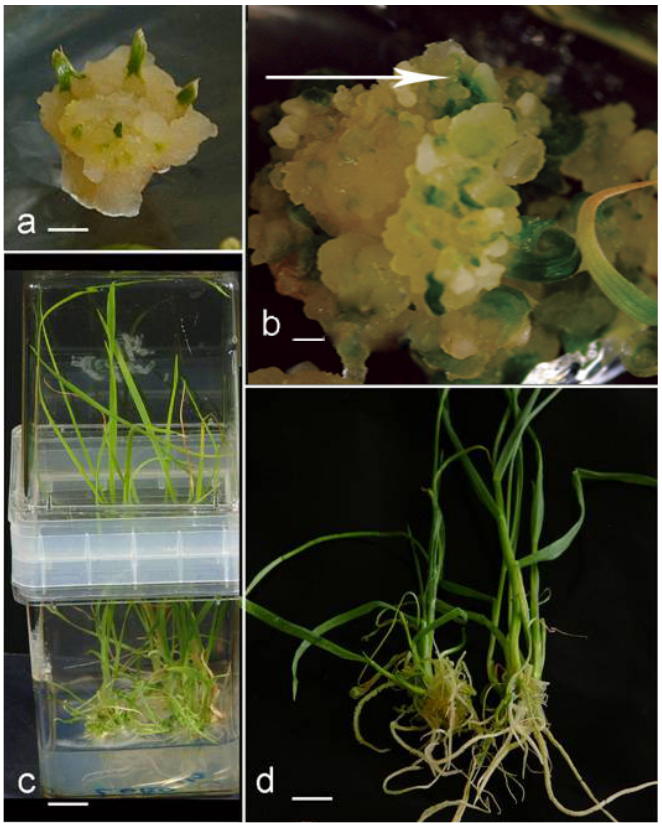

Figure 1. *In vitro* somatic embryogenesis from immature zygotic embryos of triticale. **a,b**. Callus induction and develop-ment of somatic embryos with shoot initials after 3 weeks of culture. **c.** Developing embryos transferred to MS medium for conversion of somatic embryos to plantlets. **d.** Converted plantlet/s with well developed roots in green house. Bar = 1 cm.

and developmental stage of explants on shoot regeneration has been demonstrated in a number of species. No protocol describes the effect of age of explants on *in vitro* culture of triticale. Therefore, it was considered necessary to use explants of different ages to determine the effects of age on adventitious shoot regeneration in 5 Turkish triticale genotypes.

MATERIALS AND METHODS

Plant material

Immature seeds collected from 14 - 16 and 17 - 19 days old inflorescences/spikelets after anthesis, of five hexaploid (AABBRR) genotypes ZF11, ZF12, ZF13, ZF14 and LAD388 of triticale used in the study were collected from the Department of Field Crops, Faculty of Agriculture, University of Ankara, Ankara Turkey. These were treated with 70% ethanol for 1 min followed by surface sterilization with 25% commercial bleach (Axion-Turkey, containing 5 - 6% NaOCl) supplemented with 1 drop of Tween-20 per 100 ml solution for 20 min. Thereafter, the immature seed explants were rinsed 3 times for 3 min in sterile distilled water. The caryopses were cut to remove immature embryos with a sterile surgical blade under aseptic conditions.

Culture medium

Initially, 10 immature zygotic embryos (with scutellum upward) from each genotype/accession were cultured in 4 times replicated Petri dishes® (100 x 10 mm) with 30 ml of 0.7% agar (Sigma Type A) solidified MS medium (Murashige and Skoog, 1962) supplemented with 2 mg dm^{-3} 2,4-D, 500 mg dm^{-3} glutamine, 100 mg dm^{-3} casein hydrolysate and 2% sucrose. The agar was added after adjusting pH of the media to 5.6 - 5.8 with 0.1 N KOH or 0.1 N HCl before autoclaving, at 121°C, under pressure of 118 kPa for 20 min. The Petri dishes® were sealed with Parafilm® and initially incubated in dark for two weeks at 24±2 ºC, for callus induction.

For the development of somatic embryos, the developing calli were sub-cultured on MS medium supplemented with 2 mg dm^{-3} 2,4-D, 500 mg dm^{-3} glutamine, 100 mg dm^3 casein hydrolysate, 2% sucrose and 0.7% agar without 2,4-D under light intensity of 30 000 lux in 16 h light photoperiod after two weeks of culture. The developing embryos were removed after three weeks of culture using sterilized needle and transferred to MS medium contained in Magenta GA7 vessels for maturation and recovery of plantlets. The developing plantlets were acclimatized and transferred to the greenhouse after eight weeks. The greenhouse humidity was set to 80% during early stages of development and was reduced to 40% in the later stages of acclimatization. All plants grew well in earthen pots containing sand, clay and organic matter in equal proportions.

Statistical analysis

Univariate analysis of variance was performed to determine the effect of embryo age on callus and embryo induction in 5 genotypes using SPSS 15 for Windows computer software. The differences among means were compared using Duncan's-test or t test ($p < 0.05$).

RESULTS

Callus formation

After three weeks of culture in dark, very distinct white compact embryogenic swellings were recorded on calli of immature zygotic embryos in both age groups. These were sub cultured on MS medium supplemented with 2 mg dm^{-3} 2,4-D, 500 mg dm^{-3} glutamine, 100 mg dm^3 casein hydrolysate, 2% sucrose without growth regulators in 16 h light photoperiod (Figure 1a). It appeared that genotype and age did not influence the frequency (%) of embryogenic callus formation among all genotypes and age groups statistically except for cv. LAD388 (Table 1), where frequency of callus induction from 17 - 19 days old zygotic embryos decreased sharply in genotype LAD388.

Somatic embryogenesis

All explants induced somatic embryos with distinct shoot initials (Figure 1b). Frequency (%) of embryogenesis ranged between 43.33 to 90% from 14 - 16 days old and 83.33 - 100% on 17 - 19 days old explants. Although, ZF11, ZF12, ZF14 and LAD388 genotypes showed similar response in terms of frequency of somatic embryogenesis irrespective of age, 14 - 16 days old explants

Table 1. Plant regeneration from immature embryo explants of different triticale variety and lines.

Genotype	Frequency (%) of callus formation		Frequency (%) of embryos		Mean number of somatic embryos per explant	
	14 - 16 days old zygotic embryo	17 - 19 days old zygotic embryo	14 - 16 days old zygotic embryo	17 - 19 days old zygotic embryo	14 - 16 days old zygotic embryo	17 - 19 days old zygotic embryo
Zf11	100.00	100.00aA	90.00 aA	86.67 bA	6.70 bA	3.52B
Zf12	100.00	100.00aA	86.67 bA	86.67 bA	8.07 aA	3.71B
Zf13	100.00	100.00aA	43.33 cB	93.33 bA	6.67 bA	3.30B
Zf14	100.00	100.00aA	93.33 bA	100.00 aA	6.73 bA	2.80B
LAD388	100.00	86.67bB	86.67 bA	83.33 aA	9.63 aA	3.13B

*Values within a column followed by different letters are signifiantly different at the 0.05 probability level using Dunan's multiple range test.
**Values within a row followed by different capital letters are signifiantly different at the 0.05 probability level using T test.

of genotype ZF13 were significantly inferior in inducing somatic embryogenesis (43.33%) compared to older explants with higher somatic embryogenesis (93.33%).

Age of explants (14 - 16 days or 17 - 19 days) did not affect the quality of somatic embryos and it was not difficult to establish and mature embryos to plantlets, all of which showed a sturdy growth (Figure 1c). Mean number of somatic embryos per explant ranged between 6.70 to 9.63 on 14 - 16 days old and 2.80 to 3.71 on 17 - 19 days old explants. The highest number of 9.63 somatic embryos was recorded on genotype LAD388 from 14 - 16 days old explants. All genotypes showed comparatively better performance with higher number of somatic embryos on 14 - 16 old explants. Age had inhibitory effect and 17 - 19 days old explants and showed sharp reduction in number of somatic embryos per explant in each genotype. No statistical variation existed in the number of somatic embryos per explant of all genotypes on 17 - 19 days old explants. This indicated that genotypes did not show a proportional performance in two age groups.

Acclimatization

No problem with any aberrant phenotypes was observed in acclimatization of in vitro regenerated plantlets under greenhouse conditions (Figure 1d). These plantlets matured and set seeds. A more detailed agronomic study to confirm the fidelity of the system is being carried out to establish the superiority of tissue culture raised lines.

DISCUSSION

In vitro culture response of somatic embryogenesis in plants is mainly dependent on genetic, developmental (age), environmental factors (Evans et al., 1981; Sears and Deckard, 1982; Tuberosa et al., 1982; Mathias and Simpson 1986), the explant (Redway et al., 1990), genome interaction and ploidy levels (Nakamura and Keller, 1982; Eapen and Rao, 1985; Stolarz and Lörz, 1991; Stolarz, 1991; and Barro et al., 1999). This protocol approves these findings in triticale and clearly shows that genetic and developmental factors like age of explant have clear implications on the somatic embryogenesis in triticale.

Comparing results of this study with previous reports, by Nehra et al. (1994, 1996) and Eudes et al. (2003), it can be concluded that immature tissue explants especially those derived from immature embryos or scutella could be efficiently used for tissue culture of triticales. All genotypes showed sharp variation between two age groups with induction of higher embryos on 14 - 16 days old explants.

The results of this study showed that 2,4-D could be effectively used for the creation of efficient somatic embryogenesis on immature zygotic embryos of triticale in line with previous reports by Vikrant and Rashid (2001), Przetakiewicz et al. (2003) and Birsin and Ozgen (2004) in triticale. Przetakiewicz et al. (2003) was also successful in creating somatic embryogenesis in wheat and barley using 2,4-D. 2,4-D has also been found a potent plant growth regulator for creation of somatic embryogenesis in other monocotyledons like *Paspalum scrobilatum* by Avci and Can (2006) and in *P. scrobiculatum by* Vikrant and Rashid (2001).

It is well documented that somatic embryogenesis is the result of hereditary, developmental and environmental factors (Evans et al., 1981). The results showed age dependent variable behavior of somatic embryo induction in all of the tested genotypes in two age groups. Somatic embryogenesis from immature embryos of four CIMMYT triticale varieties has also been reported on DW3 medium by Bohorova et al. (2001) with a range of 48 - 100%. They found that after six subcultures, over a 6 month period, genotypes lost their ability to regenerate plants. Vikrant and Rashid (2001) found that immature and mature zygotic embryos of hexaploid, triticale var. DT-46 formed an embryogenic callus, with subsequent somatic embryo formation upon subculture in MS or N-6 nutrient medium supplemented with various concentrations (9.0 -

22.5 µM) of 2,4-D. Of the two types of explants, embryogenic tissue from immature embryos responded at a higher frequency, to form somatic embryos over the callus surface. They found that leaf-base segment cultured on 2,4-D containing medium formed a tissue which did not form somatic embryos and instead differentiated into shoot-buds. Their results indicated that N-6 medium proved to be more effective than MS in support of somatic embryogenesis or shoot-bud formation. Przetakiewicz et al. (2003) obtained the highest values of regeneration coefficients for two triticale cultivars (Wanad and Kargo) on picloram (26.1 and 21.4 µM, respectively) and for 'Gabo' on picloram with dicamba (12.6 µM), with mean number of regenerated plantlets in range of 12 to 30. Previous studies (Nakamura and Keller, 1982; Eapen and Rao, 1985; Stolarz and Lörz, 1991; Stolarz, 1991, Immonen, 1992) also demonstrate a variable *in vitro* regeneration behavior of triticales from hexlaploids and octaploids.

Birsin and Ozgen (2004) found that out of immature embryos after 15 - 18 days of anthesis with the scutellum upwards, mature and endosperm-supported mature embryos; the endosperm-supported mature embryo was the most useful explant for plant regeneration in triticale. They found that regeneration capacity of immature embryos ranged between 53.5 to 96.3% with 5 to 13 acclimatized plants from all tested genotypes. However, Ganeshan et al. (2006), induced 34 shoots per explant from mature embryos of triticale using thidiazuron (TDZ) and 6 Benzylaminopurine (BAP) alone or in combinations.

The use of MS medium without plant growth regulators was very fruitful in maturing somatic embryos and obtaining plantlets in triticale, which is in agreement with Birsin and Ozgen (2004) and Parmaksiz and Khawar (2006). These workers also converted embryos to plantlets on plant growth regulator free MS medium. The results suggest that the age of explants have considerable implications on the somatic embryogenesis of triticale. The immature embryo culture established in the present study will be a valuable tool for functional genomics studies in triticale.

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