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Effect of polyamines on somatic embryogenesis via mature embryo in wheat

Murat AYDIN, Arash HOSSEIN POUR, Kamil HALILOĞLU*, Metin TOSUN
Department of Field Crops, Faculty of Agriculture, Atatürk University, Erzurum, Turkey

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Abstract: Plant tissue culture via somatic embryogenesis plays a key role in wheat genetic transformation studies. Therefore, the production of embryogenic calli with a high regeneration capacity is a prerequisite for efficient plant regeneration. Although immature embryos are the best type of explants for plant regeneration via somatic embryogenesis, the use of mature embryos has remarkable advantages compared to immature embryos as explants. However, the regeneration capacity of mature embryos is still lower than that of immature embryos. Plant regeneration from somatic embryos can only occur when they are mature enough. Moreover, the maturation of somatic embryos and their conversion into plantlets is closely associated with the used plant growth regulators. Polyamines are major components in the formation of somatic embryogenesis and plant regeneration. In this study, the effect of three types of polyamines on somatic embryogenesis and plant regeneration in wheat was evaluated. Although the effect of polyamines on embryogenic callus formation was not significant, their effect on responded embryogenic callus rate and plant regeneration efficiency was very significant. The highest responded embryogenic callus rate and highest regeneration efficiency were obtained on MS medium containing putrescine. Unlike other polyamines, an increase in putrescine concentration promoted a higher number of regenerated plants. The highest values for responded embryogenic callus and regeneration efficiency were determined in 1 mM concentration of putrescine. Plantlets derived from somatic embryos were successfully established in soil, producing fertile seeds.

Key words: Putrescine, spermine, spermidine, plant regeneration, wheat

1. Introduction

Plant tissue culture is the first step in genetic engineering studies (Debnath et al., 2006; Noor et al., 2009). Efficient plant regeneration systems require the successful application of biotechnology in crop improvement. One of the critical aspects of successful plant transformation in vitro is the ability to form tissues suitable for transformation and to regenerate whole plants from the transformed tissue. Immature embryos have often been used for transformation study in wheat. However, the use of immature embryos demands additional labor, time, and cost of maintaining the donor plants, especially for winter cultivars, due to vernalization requirements. Mature embryos have significant advantages compared to immature tissues as explants in in vitro culture. Therefore, mature embryos can be used as an alternative explant source of immature embryos for genetic transformation studies, because of their year-round availability and easy isolation (Patnaik et al., 2006; Ding et al., 2009; Aydin et al., 2013). Furthermore, the physiological state of mature embryos shows minimal variability (Yu et al., 2008).

Plant regeneration is correlated with the ability of embryogenic callus formation. However, plant regeneration may not occur in all somatic embryos on embryogenic callus. Aydin et al. (2013) reported that they observed a 95% embryogenic callus rate in endosperm-supported mature embryo culture, yet they obtained 50% plant regeneration from embryogenic calli. Despite all efforts, most cereal crops and wheat in particular have proven to be very tedious for the regeneration of embryogenic calli (Bouiamrine et al., 2012; Fazeli-Nasab et al., 2012). The ability of somatic embryos to be converted into plantlets depends on several factors such as embryo maturation, synthesis and accumulation of storage compounds, and development of desiccation tolerance (Xu et al., 1990; Blackman et al., 1992).

The process of somatic embryogenesis is divided into three stages according to the grade of embryo differentiation: induction of embryogenesis, proliferation and maturation of somatic embryos, and conversion of mature embryos into complete plants (Malá et al., 2009). Besides auxin and cytokinins, polyamines (putrescine, spermidine, and spermine) are also important for cellular differentiation to somatic embryogenesis (Chi et al., 1994; Bajaj and Rajam, 1996; Kevers et al., 2000). The positive promoter effect of polyamines in the conversion of

* Correspondence: kamilh@atauni.edu.tr

somatic embryos or shoot regeneration has been reported in several plant species such as *Panax ginseng* (Kevers et al., 2000), *Gossypium hirsutum* (Sakhanokho et al., 2005), *Coffea* (De-la-Pena et al., 2008), and *Citrus sinensis* (Wu et al., 2009).

The effect of polyamines on somatic embryo maturation and plant regeneration in wheat mature embryo culture has not been studied extensively. The objective of this study is to determine the effect of polyamines on plant regeneration via somatic embryogenesis in wheat.

2. Materials and methods

2.1. Plant material

Mature dry seeds of wheat cultivar Kırik (*Triticum aestivum* L.) were used as the source of mature embryos. Seeds were surface-sterilized in 70% (v/v) ethanol for 5 min, rinsed twice with sterile distilled water, incubated further in commercial bleach (5% sodium hypochlorite) with a few drops of Tween for 25 min, and rinsed twice in sterile distilled water. The seeds were imbibed in sterile water for 16 to 17 h at 4 °C in the dark. The shoot axes of mature embryos as well as their scutellae were cut both longitudinally and horizontally without being detached from the seeds. Prepared seeds were cultured on callus induction medium.

2.2. Callus initiation

Prepared endosperm-supported mature embryos were transferred to the callus induction medium containing MS basal salts and vitamins (Murashige and Skoog, 1962), supplemented with 12 mg/L dicamba, 0.5 mg/L IAA, 20 g/L sucrose, 7 g/L agar, and 1.95 g/L MES. All media were adjusted to pH 5.8 with 1 N NaOH. Media solutions containing basal salts and solidifying agent were autoclaved at 121 °C for 15 min for sterilization. Vitamins and plant growth regulators were filter-sterilized. Explants were cultured at 25 °C in the dark, and 21 days later the callus induction (%) was measured. The developed calli were then separated from the seeds.

2.3. Embryogenic callus formation and maintenance

For the formation and maturation of embryogenic calli, the primary calli, with or without embryoids, were transferred to an MS medium with different concentrations (0, 0.5, and 1.0 mM) (Kelley et al., 2002) of three different kinds of polyamines (putrescine, spermidine, and spermine), 20 g/L sucrose, 7 g/L agar, and 1.95 g/L MES hydrate at 25 ± 1 °C in the dark for 14 days. The embryogenic callus rate (%) was calculated at the end of this culturing period.

2.4. Plant regeneration

Embryogenic calli were transferred to MS medium containing 0.5 mg/L TDZ (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, Thidiazuron), 20 g/L sucrose, and 7 g/L agar for plant regeneration, and were incubated for 30 days under a 16-h light (light intensity of 62 μmol m^{-2} s^{-1}) and 8-h dark photoperiod at 25 °C. Responded embryogenic callus and regeneration efficiency (number of regenerated plants per explant) rates were determined after 4 weeks. The regenerated plantlets were transferred to magenta boxes containing the same regeneration medium and were grown under the same plant regeneration conditions until they reached 10–12 cm in height. Plantlets were transplanted to soil and acclimatized. Acclimatized plants (of which 75% survived) were grown under greenhouse conditions to maturity.

2.5. Statistical analysis

This study was carried out in a complete randomized experimental design of 3 × 4 factorial arrangements with 4 replicates. Each petri dish was considered as an experimental unit and 10 mature embryos were cultured in each petri dish. Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure in SAS (SAS Institute, 1996). Means treatments and interactions were compared using Fisher's least significant difference (LSD) test.

3. Results

3.1. Callus and embryogenic callus formation (%)

Callus formation induced by the callus initiation medium was recorded as 100% in endosperm-supported mature embryo culture (Figure 1A).

Both embryogenic and nonembryogenic calli were observed in the embryogenic callus initiation medium. The embryogenic callus was characterized by compact presence of globular somatic embryos and a light yellow color (Figure 1B), whereas the nonembryogenic callus was characterized by white color and a watery appearance. The Table shows that the percentage of embryogenic callus formation ranged from 90% to 100%, based on polyamine types and their concentrations. The main effect of polyamines on embryogenic callus formation was not significant; however, embryogenic callus formation was significantly affected by polyamine concentration ($P < 0.05$). In addition, there was no significant interaction between polyamine and concentrations (Table).

When comparing the effects of polyamines on embryogenic callus formation, the highest mean embryogenic callus rate was observed in putrescine at 100%. In other words, putrescine has high potential to stimulate embryogenic callus production, whereas the lowest embryogenic callus formation was in spermine, at 96.7% (Table). Based on concentration levels, the control (0 dose) and 0.5 mM treatments gave the highest mean embryogenic callus formation (100%), whereas the lowest mean embryogenic callus rate was observed in the treatment of 1 mM (Table), at 95%. Embryogenic callus formation had the tendency to decrease when the

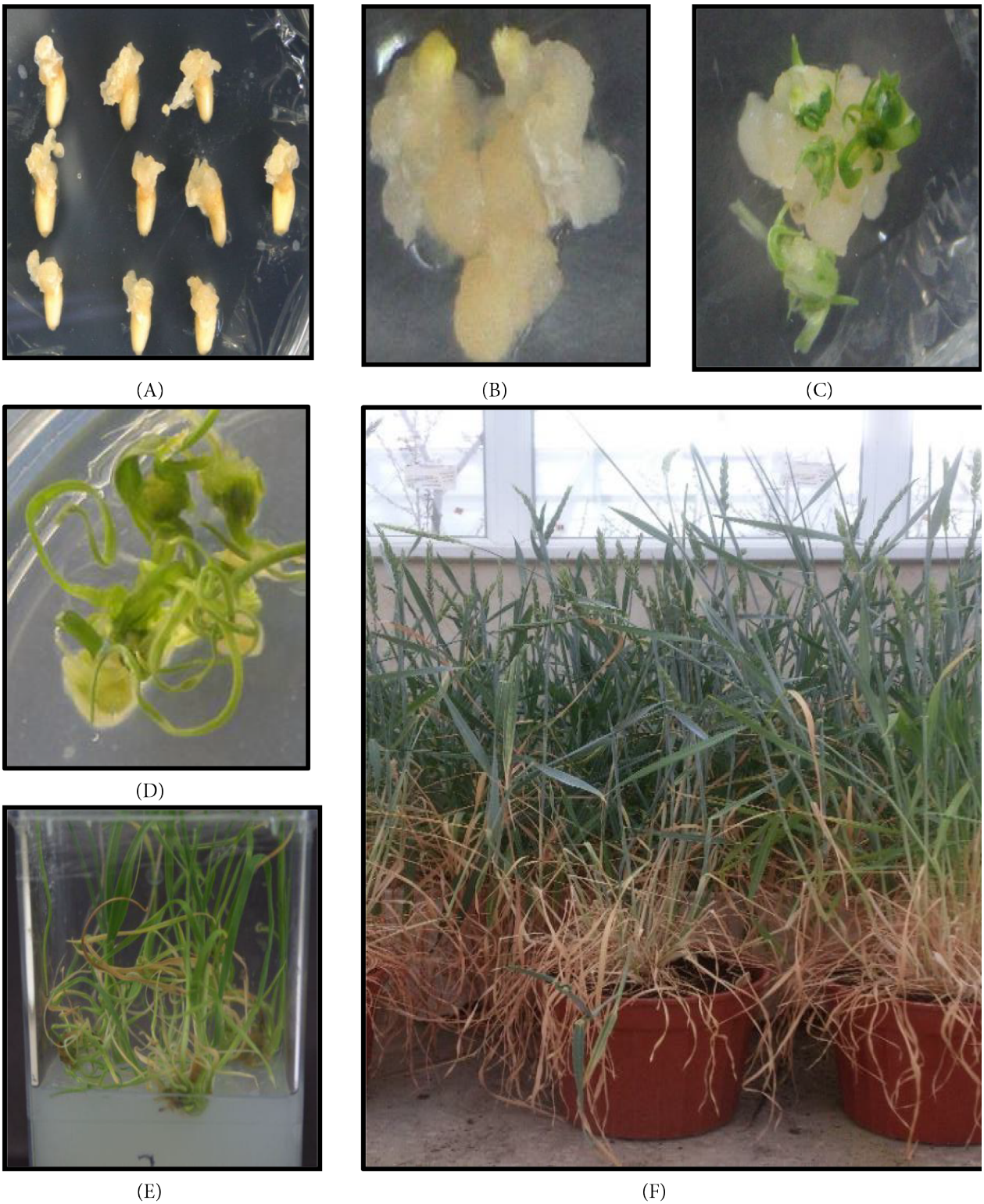

Figure 1. Callus formation, somatic embryogenesis, and plant regeneration from endosperm-supported mature embryos in wheat: (A) callus formation; (B) somatic embryos on embryogenic callus; (C) responded embryogenic callus; (D) plant regeneration; (E) plantlets in Magenta boxes; and (F) regenerated plants in soil.

Table. Effect of different polyamine types and their concentrations on embryogenic callus rate, responded embryogenic callus rate, and regeneration efficiency from endosperm-supported mature embryo-derived calli of *Triticum aestivum* L. var. Kırik.

Polyamine type	Concentrations (mM)	Embryogenic callus rate (%)[a]	Responded embryogenic callus rate (%)[b]	Regeneration efficiency (number of regenerated plants per explant)[c]
Putrescine	0.0	100	65.0	2.5
	0.5	100	80.0	2.3
	1.0	100	90.0	2.9
	Mean	100	78.3	2.5
Spermidine	0	100	65.0	2.5
	0.5	100	72.5	1.5
	1.0	95.0	73.3	1.0
	Mean	98.3	70.3	1.7
Spermine	0.0	100	65.0	2.5
	0.5	100	60.0	1.3
	1.0	90.0	51.8	1.0
	Mean	96.7	58.9	1.6
Polyamine mean	0.0	100	65.0	2.5
	0.5	100	70.8	1.7
	1.0	95.0	71.7	1.6
	Mean	98.3	69.2	1.9
F value (polyamine) (P)		1.29^{ns}	40.07**	89.92**
F value (concentration) (C)		3.86*	5.63**	95.18**
F value (P × C)		1.29^{ns}	12.92**	23.17**
Polyamine $LSD_{(0.05)}$		-	4.5	0.2
Concentration $LSD_{(0.05)}$		4.3	4.5	0.2
P × C $LSD_{(0.05)}$		-	7.7	0.3
Coefficient of variation (%)		5.2	7.7	10.3

**: Significant at $P < 0.01$, *: Significant at $P < 0.05$, ^{ns}: Nonsignificant at $P > 0.05$.
[a] (Embryogenic callus number/explant number) × 100.
[b] (Responded embryogenic callus number/explant number) × 100.
[c] (Regenerated plant number/explant number).

concentration of spermine and spermidine was increased from 0.5 mM to 1 mM.

3.2. Responded embryogenic callus rate

Plant regeneration may not occur in all somatic embryos on the embryogenic callus. Therefore, only the responded embryogenic callus rate was evaluated in study. Responded embryogenic callus was characterized as embryogenic callus-forming plantlets that have healthy roots and shoots (Figure 1C). Analysis of variance showed that the responded embryogenic callus rate was significantly ($P < 0.01$) affected by polyamine, concentration, and their interaction (Table). Putrescine (78.3%) gave the best responded embryogenic callus rate among polyamines and was followed by spermidine (70.3%) and spermine (58.9%).

A significant difference was observed among polyamine concentrations. Based on concentration levels, the control (0 mM) treatment gave the lowest mean responded embryogenic callus formation (65%), whereas the highest mean responded embryogenic callus formation was observed in the treatment of 1 mM, at 71.7% (Table). The responded callus rate increased with the elevation of polyamine application from 0 to 1 mM.

The results indicated that changes in the effect of polyamines were dependent on concentration. The elevation in concentration of putrescine and spermidine increased the responded embryogenic callus rate, whereas the embryogenic callus rate decreased when the concentration of spermine increased from 0 mM to 1 mM. The 1 mM putrescine treatment was determined as the most effective polyamine type and the concentration for responded embryogenic callus rate.

3.3. Regeneration efficiency (number of regenerated plants per explant)

The mean number of regenerated plants was influenced by the polyamine type, concentration, and their interaction. The mean number of regenerated plants per explant ranged from 1.6 to 2.5 based on polyamine type. Among the polyamines, the highest number of regenerated plants (2.5) per explant was observed on the culture medium containing putrescine.

Regeneration efficiency was strongly influenced by polyamine concentrations. The control (0 mM) treatment gave the highest mean regeneration efficiency (2.5 plants), whereas the lowest mean regeneration efficiency, with 1.6 plants, was observed in the treatment of 1 mM (Table).

When the data were analyzed in terms of polyamine type and concentrations, it was noted that the rise in concentrations of putrescine increased the number of regenerated plants, whereas the rise in concentrations of the other polyamines from 0.5 mM to 1 mM caused a decrease. Mature embryos showed the best response, with 2.9 regenerated plants per explant on culture medium containing 1.0 mM putrescine (Figures 1D and 1E). Regenerated plants were established into normal plants in the greenhouse and set seed (Figure 1F).

4. Discussion

Crop improvement through genetic transformation in wheat depends on an efficient regeneration system through somatic embryos. Polyamines, including putrescine, spermidine, and spermine, are present in all plant cells (Galston, 1983) and play a role in cell division and differentiation (Basu et al., 1997). Polyamines have been indicated as playing a vital role in organogenesis and somatic embryogenesis (Minocha and Minocha, 1995; Cohen, 1998). Spermidine and spermine are associated with differentiation, whereas putrescine is involved in cell divisions (Minocha et al., 1999; Niemi et al., 2002).

The effect of polyamines on somatic embryogenesis and organogenesis has been studied by several researchers (Chi et al., 1994; Bajaj and Rajam, 1996; Ha et al., 1998), who suggested that polyamines function as regulators of somatic embryos. Thiruvengadam et al. (2013) reported that polyamines have an enhancement effect during the early stages of embryogenic callus growth in terms of the number of somatic embryos in *M. dioica*.

It was found that putrescine treatment enhanced responded embryogenic callus rate and regeneration efficiency in our study. Dewi et al. (2001) reported that putrescine was more efficient than spermidine and spermine in callus induction and plant regeneration in rice anther culture. Similar observations were reported in several plant species such as *P. ginseng* (Kevers et al., 2000), *G. hirsutum* (Sakhanokho et al., 2005), *M. charantia* (Paul et al., 2009), and *Daucus carota* (Gorecka et al., 2014).

When polyamine types and concentrations were evaluated in our study, it was determined that 1 mM putrescine treatment enhanced responded embryogenic callus rate and regeneration efficiency. Furthermore, exogenously supplied spermine and spermidine inhibited somatic embryogenesis and plant regeneration. Similar results were also reported by Dewi et al. (2001) and Dewi and Purwoko (2008). Paul et al. (2009) studied the influence of putrescine (0.1, 0.5, and 1 mM), spermidine (0.1, 0.5, and 1 μM), and spermine (0.1, 0.5, and 1 μM) on somatic embryogenesis in *M. charantia* L. and found that 1 mM putrescine concentration was the most effective in terms of somatic embryogenesis, followed by 0.1 μM concentration of spermine and spermidine, which also supports our findings. The results of both studies showed that spermine and spermidine should be used in much lower doses than putrescine to enhance somatic embryogenesis and plant regeneration.

Plantlet formation from somatic embryos depends on factors such as somatic embryo maturation, synthesis and accumulation of storage compounds, and development of dehydration tolerance (Xu et al., 1990; Blackman et al., 1992). However, the influence of polyamines on somatic embryogenesis is not clear (Niemi et al., 2002). Santanen and Simola (1992) stated that polyamines may play a role in the accumulation of seed storage protein and embryo maturation in *Picea abies* L. Furthermore, the initiation and proliferation of embryogenic cultures are generally achieved by exogenous application of auxin (Attree et al., 1991), whereas abscisic acid (ABA) is emphasized as an important component in both the formation of somatic embryogenesis and in embryo (somatic and zygotic) maturation (Angoshtari et al., 2009). ABA encourages the accumulation of reserve substances and maturation

and reduces the frequency of abnormal morphologies and secondary embryogenesis (Garcia-Martin et al., 2005). The role of ABA may actually stimulate the synthesis of polyamines. Alcázar et al. (2006) reported that ABA is responsible for the induction of genes encoding for enzymes in the biosynthetic pathway of polyamines in *Arabidopsis*. Additionally, the level of putrescine was found to be high in ABA-containing medium in *Picea abies* (Santanen and Simola, 1992). These results may confirm that polyamines are mainly responsible for the maturation of somatic embryogenesis.

In vitro conditions can be very stressful for plant cells, and this stress may encourage somaclonal variation (Kaeppler and Phillips, 1993; Shepherd and Dos Santos, 1996). This variation affects the use of tissue culture negatively in genetic transformation studies. Exogenous application of putrescine has been successfully used to enhance salinity (Chattopadhayay et al., 2002), cold (Nayyar and Chander, 2004), drought (Zeid and Shedeed, 2006), water logging (Arbona et al., 2008), and flooding tolerance of plants (Yiu et al., 2009). Polyamines are small, positively charged aliphatic amines at cellular pH values. Owing to their polycationic nature, they are charged with anionic macromolecules such as DNA and RNA, as well as with negative groups of membranes (Malá et al., 2009). Polyamines may protect DNA from reactive oxygen species (Ha et al., 1998). Polyamines induce DNA stabilization and could probably reduce somaclonal variation. However, this polyamine feature should be confirmed by molecular marker studies. In vitro culture conditions, as well as many stresses, are associated with increased accumulation of reactive oxygen species. Polyamines may protect DNA from reactive oxygen species (Ha et al., 1998).

In this research, it was determined that putrescine plays a key role in somatic embryogenesis in mature embryo cultures of wheat. This is the first study that investigates and evaluates polyamines in mature embryo cultures of wheat.

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