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EFFECT OF PLANT GROWTH REGULATORS ON *IN VITRO* PLANT REGENERATION OF WHEAT (*Triticum aestivum* L.) FROM EMBRYO EXPLANTS

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ABSTRACT

An efficient method was developed for multiple shoot regeneration of Albanian wheat (*Triticum aestivum* L.) cultivar "Dajti" from mature embryos without callus formation, for a short period of time. The effect of explants, isolated shoot embryonic meristem and shoot embryonic meristem with scutellum and various combinations of plant growth regulators in MS media on direct shoot regeneration of wheat was investigated. Among different combination of 2,4-D, IBA, NAA and BAP tested, embryo explants cultured in MS medium supplemented with 2 mg/L BAP, 0.6 mg/L 2,4-D resulted as the most efficient direct shoot regeneration and produces a maximum of 2.5 shoot per explants and 3.4 cm shoot length. Plantlets were successfully transferred to rooting medium. The greatest mean numbers of roots were obtained on MS media supplemented with 0.5 mg/L NAA, and the best mean value of root length (8.2 cm) at 0.5 mg/L IBA. In this paper by simple manipulating the concentrations of BAP, 2,4 D, IBA and NAA in the culture medium, is described a method for rapidly obtaining whole plants of wheat without the subculture, using embryo explants.

Keywords: embryo, direct shoot regeneration, growth regulators, rooting.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the leading cereals in the world. In Albania it is the most important cereal crop and major staple food grown all over the country. Conventional breeding programs in Albania are used to enhance the production and quality improvement of wheat crop. However, limited gene pool availability and long duration of these methods are the major limitations for improvement of the crop through conventional methods (Hamid and Sadaf, 2014).

Genetic engineering techniques are gaining popularity because the desired gene can be introduced from any source without species barrier in wheat genome in short time to improve its characters (Malik *et al.,* 2003). Transformation of wheat crop entirely depends upon regeneration of transformed explants through tissue culture (Yu *et al*., 2008). Consequently, establishment of reliable tissue culture protocols for plant regeneration is desired in order to improve wheat yield (Noor *et al*., 2009) through genetic transformation. Development of efficient plant regeneration protocol from either single cell or organized tissue is important for many commercially important crops like wheat the common source of energy and proteins for the world population (Hamid and Sadaf, 2014). Tissue culture of wheat depends upon genotype of wheat (Mahmood *et al*., 2012), culture medium (Mathias and Simpson, 1986; Mahmood *et al*., 2012) and growth regulators (Saad *et al*., 2004). *In vitro* regeneration of wheat is possible from different explants such as mature and immature embryos, seeds, endosperm, leaves, shoot bases and root tips (Sarker and Biswas, 2002). Among them the immature embryo was reported as the best for callus induction and shoot regeneration (Sarker and Biswas, 2002). But availability of immature embryo is limited by wheat growing season or requires expensive and sophisticated growth chambers. On the other hand, mature seeds of wheat are readily available throughout the year, hence can be used for plant regeneration in any convenient time.

The present study was conducted to develop efficient *in vitro* direct shoot regeneration by using mature embryos as explants sources. Objective of this research was tissue culture response of wheat explants grown on culture medium supplemented with different concentration of plant growth hormones. The results of the present study will be helpful for wheat breeding program through tissue culture technique and maintenance of desire traits in wheat, also to avoid the risk of soma clonal variation often observed in genetic manipulation process.

MATERIALS AND METHODS

Experimental site and source of explants: This study was carried in the Laboratory of Plant Tissue Culture of the Institute of Plant Genetic Resources, Agriculture University of Tirana during 2016. Seeds of Albanian wheat (*Triticum aestivum* L.) cultivar "Dajti" used for this research are part of the seed collection of this Institute.

Seeds sterilization: Mature seeds of selected wheat cultivar were washed under running tap water with detergent. Then they were disinfected with 75% ethanol

for 30 seconds followed by 30% Clorox for 10 minutes with continuous shaking under laminar flow. Seeds were washed several times with autoclaved water to remove the sterilant.

Plant media: The mature embryos were aseptically excised from the caryopses, and then dissected to two types of explants including cut shoot embryonic meristem and shoot embryonic meristem with scutellum (Fig. 1).

Every explant was gently removed from the embryos and inoculated on Petri dishes with MS medium supplemented with different concentrations of plant hormones. The pH media was adjusted to 5.8±0.1 before autoclaving at 121^0C for 15 minutes. Cultures were kept in growth room at temperature of 25 ± 1^0C for 2 weeks at photoperiod 8/16 h. After this period wheat explants were transferred at fresh medium for further proliferation and growth up to one week. After proliferation explants were transferred at regeneration media MS with different levels of BAP (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/L) in combination with IBA (0.5 and 1.5 mg/L), NAA (1.5 and 2.5 mg/L) and 2.4-D (0.4, 0.6, 0.8 mg/L) to get maximum regeneration and shoot induction. Each medium was supplemented with 3% sucrose, 1.5 mg/L myo-inositol, and vitamins. Media were solidified with 6 g/L agar. Plantlets were cultured at MS strong media with different concentration of auxin IBA (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) and NAA (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L; Table 1).When roots were developed plants were transferred in soil filled pots. Data were collected for shoot sprout frequency, shoot number, shoot length, leaves number, root number and root length.

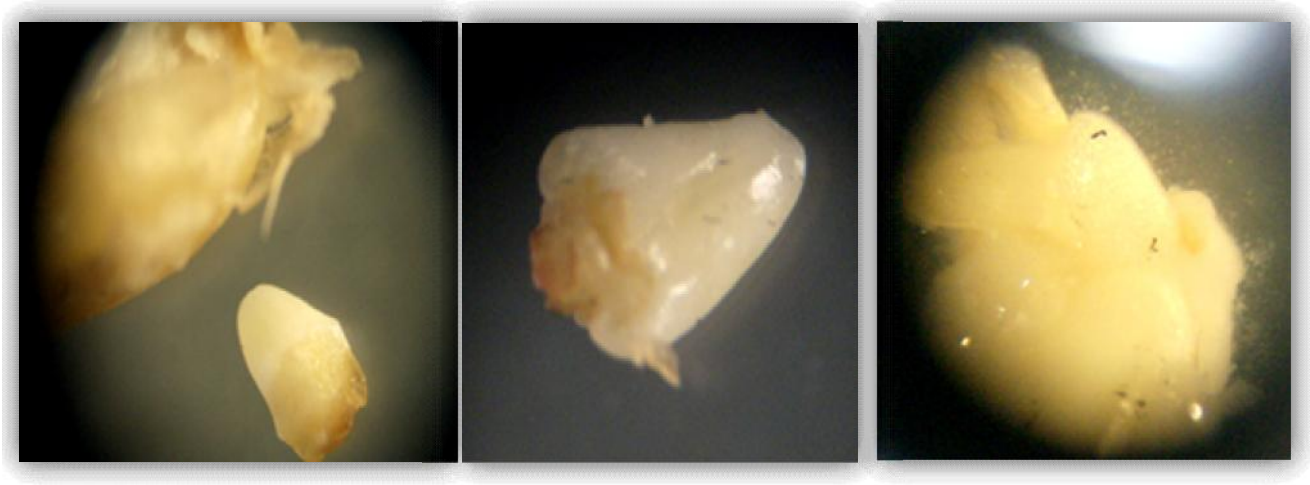

Figure 1. Embryo explants aseptically excised from the caryopses at wheat (*Triticum aestivum* L.) "Dajti" cultivar.

Table 1.**Plant growth regulators used for shoot induction and wheat plant regeneration.**

Media		Plant hormones mg/L			
		BAP	2,4-D	NAA	IBA
M_1	MS	1.0	0.2	0.0	0.0
M_2	MS	1.5	0.4	0.0	0.0
M_3	MS	2.0	0.6	0.0	0.0
M_4	MS	2.5	0.8	0.5	0.5
M_5	MS	3.0	0.0	1.5	1.5
M_6	MS	3.5	0.0	2.5	1.5

Statistical analysis: Statistical analysis was set as completely randomized design. Data were statistically analyzed using ANOVA table on excel program and presented as average ± standard error. Means were separated according to Duncan's multiple Range Tests (Duncan, 1955) at 5% probability level.

RESULTS AND DISCUSSION

Effect of plant growth regulators on regeneration: Auxins and cytokinins are the most common plant growth regulators used *in vitro* culture of plant tissues (George and Debergh, 2008). They turn the explants to produce a direct multiple shooting response (Malik *et al*., 2007), are important in cell division in differentiation stages and promoting shoot induction (Ganeshan *et al*., 2006).

Reference (Ahmad *et al*., 2002) used a culture medium containing BAP and 2,4-D in meristem multiplication system for spring wheat genotype.

In this study the combination of BAP (2 mg/L) and 2, 4-D (0.6 mg/L) was the best auxin/cytokinin ratio to conduct direct shoot regeneration. After two weeks of maintenance in proliferation media, two types of embryonic explants used were transferred to regeneration medium with different levels of hormones. The survival rate was high (80%). According to the results of this study mature explants, cut shoot embryonic meristem and shoot embryonic meristem with scutellum derived from *Triticum aestivum* L. embryos produced multiple shoots directly without intermediate callus phase on MS medium. Yasmin *et al*. (2001) observed that the mature embryos failed to initiate any type of callus at low concentrations of 2,4-D resulting only in initial swelling. Elimination of intermediate callus phase is exclusive concession that used in rice (Nhut *et al*., 2007), cereal (Eudes *et al*., 2003) to decrease the time, frequency of soma clonal variation and especially genotype dependency (Sharma *et al*., 2004).

There was no significance effect of explants type on shoot regeneration in this study. Meristem section is one of the most important prerequisites for direct shoot regeneration. It was observed direct shoot regeneration without intermediate callus phase in all the explants after a short period (10 days). Isolated shoot embryonic meristem and shoot embryonic meristem with scutellum explants gave no significant differences in shoot regeneration, emphasizing the idea that the meristematic tissues have rapid cell division rate and represents a high potential for multiplication and regeneration, showing less variability in genotypic and phenotypic response, respectively (Eudes *et al*., 2003).

Data given in Table 2 suggest that the explants exhibited good response for regeneration in MS strong medium. Same results are reported from other authors (Raziuddin *et al*., 2010; Mahmood *et al*., 2012). They concluded that wheat cultivars have best response and perform better on MS while in other type of media used. Media was solidified with 6 g/L agar. The role of agar and its concentration on shoot regeneration has been object of many studies (Ali *et al*., 2004; Mahmood *et al*., 2012). It is thought that agar has agro pectin with its sulphate side groups and with some other organic impurities due to which it might have inhibitory effects on callus proliferation (Bhojani and Razdan, 1996).

The utility of BAP to induce multiple shoot formation was analyzed in a variety of experimental approaches to establish *in vitro* regeneration protocols using embryonic explants (Ahmad *et al*., 2002; Schulze, 2007). The results obtained in this study are consistent with those reported from Ahmad *et al*. (2002), where it is indicated that the higher values of BAP hormone in the media is capable of stimulating the differentiation of adventitious buds from shoot apices. In this study, the maximum values of wheat plants biological parameters were observed in MS medium supplemented with 2 mg/L BAP (Fig. 2; 2.5±0.2 shoot number; 3.4±0.5 cm shoot length and 2.6±0.3 leaves number).

Rooting results: The micropropagated shoots were successfully rooted in full MS medium supplemented with either IBA or NAA concentrations. The greatest mean numbers of roots (3.1±0.2) were obtained on MS supplemented with 0.5 mg/L NAA. The highest value of root length (8.2±0.5), however was produced in IBA concentration (0.5 mg/L).

The results obtained for rooting of the "Dajti" cultivar plantlets in MS media supplemented either with IBA or NAA goes in agreement with many studies. Two types of auxin used in this study have been previously, successfully reported for inducing *in vitro* rooting in a variety of plants (Ramanayake *et al*., 2006; Mishra *et al*., 2008; Yasmin *et al*., 2009).

Table 2. **Effect of plant growth hormones on multiple shoot induction of *Triticum aestivum* L. "Dajti"cultivar after 6 weeks of culture.**

Media	Shoot sprout frequency, %	Shoot number (mean ±SE)	Shoot length, cm (mean ±SE)	Leaves number (mean ±SE)
M_1	65	1.0 ± 0.3^{c}	2.8 ± 0.6^{bc}	2.4 ± 0.3^{a}
M_2	83	1.9 ± 0.2^{b}	3.2 ± 0.6^{ab}	2.1 ± 0.4^{b}
M_3	90	2.5 ± 0.2^{a}	3.4 ± 0.5^{a}	2.6 ± 0.3^{a}
M_4	85	2.1 ± 0.2^{b}	1.9 ± 0.6^{e}	1.7 ± 0.4^{bc}
M_5	70	1.4 ± 0.5^{c}	2.0 ± 0.5^{dc}	1.4 ± 0.3^{d}
M_6	62	1.7 ± 0.3^{bc}	2.5 ± 0.3^{cd}	1.6 ± 0.4^{cd}

Means with the same letter (s) in the same column are not significantly different at 5% using Duncan Multiple Range Test

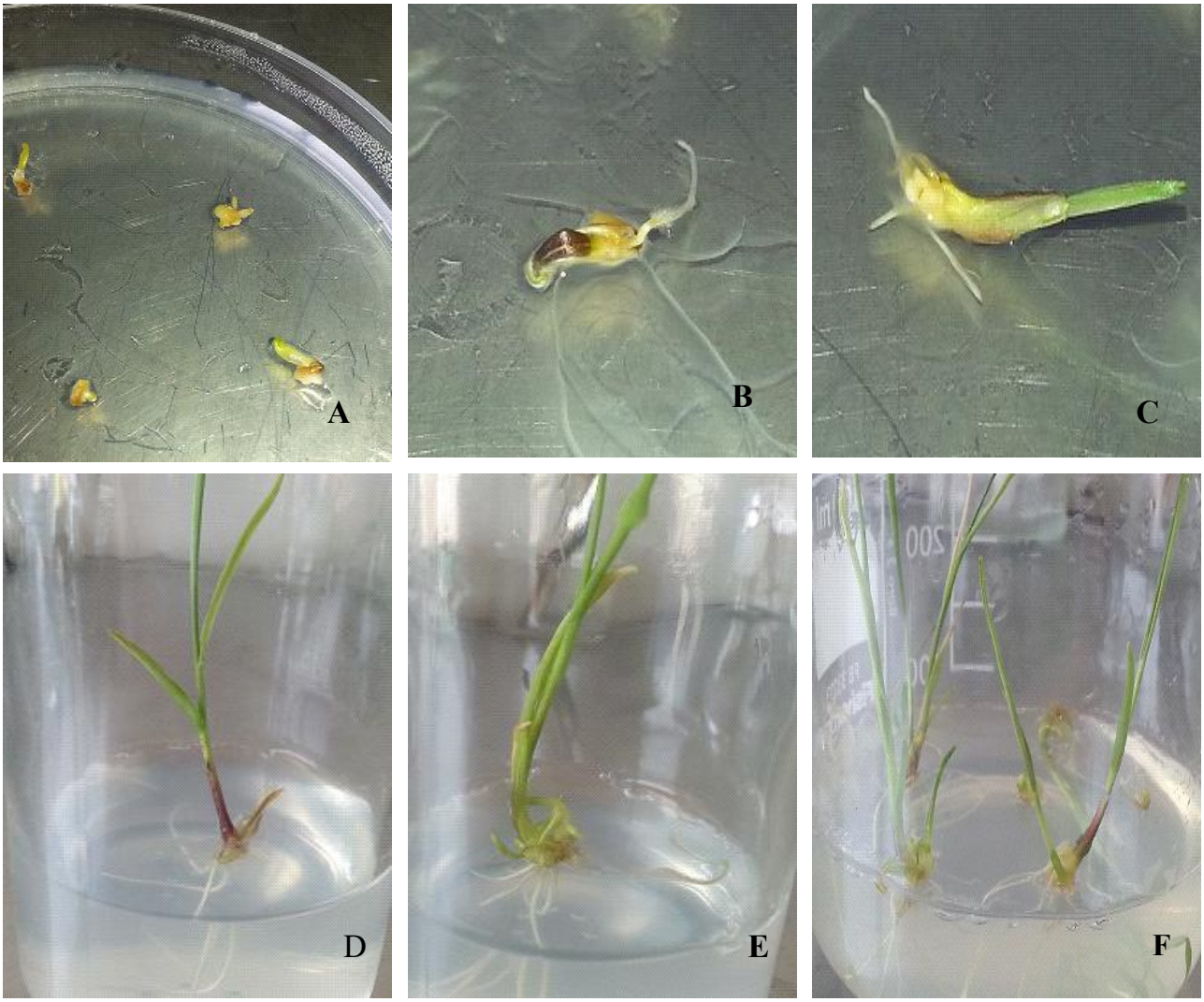

Figure 2. Proliferation of wheat (*Triticum aestivum* L.) "Dajti" cultivar shoot embryonic meristem with scutellum on M3 medium. A) after 5 days of culture. B) after 10 days and C) after 13 days of culture D) regenerated plantlets on jar in M1 medium after 6 weeks. E) on M5 and F) shoot multiple formation after 6 weeks on M3 medium.

Table 3. Effect of auxin type on rooting of wheat regenerated plantlets after 6 weeks of culture.

Type of auxin	Auxin concentration mg/L	Root number (mean ± SE)	Root length, cm (mean ± SE)
IBA	0.5	1.3±0.2^{c}	8.2±0.5^{a}
IBA	1.0	1.4±0.2^{c}	7.7±0.6^{b}
IBA	1.5	1.4±0.3^{c}	6.9±0.6^{c}
IBA	2.0	1.2±0.2^{c}	5.6±0.8^{d}
NAA	0.5	3.1±0.2^{b}	4.7±0.4^{e}
NAA	1.0	3.7±0.2^{a}	2.1±0.4^{f}
NAA	1.5	3.5±0.3^{a}	1.9±0.2^{f}
NAA	2.0	3.3±0.3^{ab}	1.8±0.4^{f}

Means with the same letter (s) in the same column are not significantly different at 5% using Duncan Multiple Range Test

Figure 3. Wheat (*Triticum aestivum* L.) of "Dajti" cultivar plants after acclimatization.

The regenerated shoots were established well in the soil (Fig. 3), the new wheat plants didn't show any morphological abnormalities and set seeds normally.

Conclusions: The present study was conducted to develop efficient *in vitro* direct shoot regeneration by using mature embryos as explants sources. Objective of this research was tissue culture response of wheat embryo explants grown on culture medium supplemented with different concentration of plant growth hormones.

Direct shoot regeneration is important since fewer soma clonal variations are likely to arise in indirect regeneration method. Meristematic section is one of the most important prerequisites for direct shoot regeneration. In this study it was observed direct shoot regeneration without intermediate callus phase, from isolated shoot embryonic meristem and shoot embryonic meristem with scutellum with various combinations of plant growth regulators in MS media, after a short period (10 days). Among different combination of 2,4 D, IBA, NAA and BAP tested, embryo explants cultured in MS medium supplemented with 2 mg/L BAP, 0.6 mg/L 2,4 D resulted in the most efficient direct shoot regeneration and produces a maximum of 2.5 shoot per explants and 3.4 cm shoot length. Plantlets were successfully transferred to rooting medium. The greatest mean numbers of roots were obtained on MS media supplemented with 0.5 mg/L NAA, and the best mean value of root length (8.2 cm) at 0.5 mg/L IBA. In this paper by simple manipulating the concentrations of BAP, 2,4 D, IBA and NAA in the culture medium, is described a method rapidly obtaining whole plants without the subculture of wheat using embryo explants.

The results of the present study will be helpful for wheat breeding program through tissue culture technique and maintenance of desire traits in wheat, also to avoid the risk of soma clonal variation often observed in genetic manipulation process.

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