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The effect of medium composition on callus induction and plant regeneration frequencies from mature embryos of wheat cultivars with various resistance to *Parastagonospora nodorum*

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Abstract

Bread wheat (*Triticum aestivum* L.) is one of the three most important cereal crops of the world and it is grown under a variety of pedo-climatic conditions. The crop suffers from many fungal pathogens, and especially from *Parastagonospora nodorum (Berk.) Quaedvlieg, Verkley & Crous.*, which represents the major deleterious biotic stress factor to the cereal species in question. Parastagonospora (syn. Stagonospora, Septoria) nodorum blotch (SNB) is a disease of wheat (*T. aestivum* L.) elicited by this necrotrophic fungus on all green plant parts. It reduces assimilative area of glumes, peduncles, stems, leaf blades and sheaths what affects adversely quantity and quality of grain yield. Among wheat cultivars complete resistance to *P. nodorum* is not encountered. The effect of resistance breeding by conventional methods may be supported by biotechnological tools, e.g. by somatic embryogenesis or androgenesis. This study was undertaken to improve callus induction and plant regeneration from mature embryos of five winter wheat cultivars with various resistance levels to *P. nodorum*. For the purpose three type of auxins [2,4 dichlorophenoxyacetic acid (2,4-D); 3,6-dichloro-o-anisic acid (dicamba); 1-naphthaleneacetic acid (NAA)], and the effect of maltose vs. sucrose were evaluated. The results demonstrated relatively high embryogenic potential of all winter wheat cultivars used in the study. Inducing media supplemented with dicamba and sucrose were the most suitable for embryogenic callus formation, however, the highest efficiency of plant regeneration was obtained on medium without phytohormones.

Key words: Somatic embryogenesis, wheat *in vitro* culture, plant regeneration, mature embryo, auxins

Introduction

Successful plant regeneration from cells, tissues and organs is one of the important steps in the application of biotechnology to agronomic traits improvement including quality and resistances to biotic and abiotic stresses. Wheat is one of the most common species of food and feed crops and it is a fundamental nutrient source of human calories worldwide. Plant regeneration frequencies from *in vitro* cultures of the species are generally low. Researchers still strive to optimize the callus induction procedures and efficiency of plant regeneration for wheat mature embryos.

Somatic embryogenesis is the process in which a bipolar structure (containing both shoot and root apical meristems differentiated simultaneously at opposite poles) is formed

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from a single somatic cell or from a group of somatic cells (Rocha and Dornelas; 2013). Despite many limits, immature embryos are the most frequently used explants source for wheat *in vitro* culture. Zhou and Lee (1983) were the first to obtain successful plant regeneration from mature embryos as an alternative to immature embryos. They observed remarkable advantages: mature embryos are easy to handle, there is no time limitation and they are available in bulk quantities. Unfortunately, the regeneration efficiency from mature embryos is relatively low and strongly associated with plant genotype (Ozgen et al. 1998). In order to improve *in vitro* culture efficiency, many factors affecting callus induction and plant regeneration have been studied widely in wheat mature embryo culture. The ability of wheat callus induction and plant regeneration are influenced by the explant source (Redha and Talaat 2008, Liu et al. 2008) medium (Przetakiewicz et al. 2003) and genotype (Filippov et al. 2006, Rakoczy-Trojanowska and Malepszy 2005). Efficiency of wheat somatic embryogenesis was mainly compared with dicamba 2,4-D, picloram and NAA (Ren et al.; 2010, Ayse et al.; 2006, Mendoza and Kaeppler; 2002). Majority of literature publications concerning cereal regeneration report culture 2,4-D as the main growth hormone inducing embryogenic callus. Zhou and Lee (1983) demonstrated a significant effect of the type and doses of auxins on optimization of wheat mature embryo culture. They compared the effects of 13 auxins on callus induction and growth of wheat cultivars 'Chinese Spring' and 'Frederick'. Another one of the important factors for callus induction and plant regeneration is the optimal sugar source. In the induction culture media, sucrose and maltose are the most commonly used carbon sources in wheat (Orsinky et al. 1990). Mendoza and Kaeppler (2002) compared the effects of different auxins and carbon sources on callus induction of the wheat cultivar Bobwhite. The latter authors proved significant role of sugar type to improve the rates of callus induction and plant regeneration.

Based on the above described considerations, the objective of this study was to determine the optimum conditions for effective callus induction and plant regeneration. For the purpose the effects of three types of auxin and carbon sources to induce embryogenic callus from mature embryos of six winter wheat cultivars with various resistance to Parastagonospora nodorum blotch were compared.

Materials and methods

Plant material and explant preparation : Six winter wheat cultivars with various resistance to *P. nodorum* were used as the source for mature embryo culture. Assess the level of infection by pathogenic fungus in 9-digit scale was carried out (Table 1.).

Table 1. Levels of *P. nodorum* infection of leaves and glumes of six winter wheat cultivars, donors of mature embyos, as assessed on 9-digit scale (susceptible; ± moderately susceptible/resistant; + resistant).

WHEAT CULTIVARS	LEAVES	GLUMES
Arkadia	-	-
Astoria	-	-
Bamberka	+	+
Muza	±	±
Ostroga	+	+
Wydma	±	±

The seeds of donor plants were surface-sterilized sequentially with 70% ethanol for 5 min and 10% commercial bleach (5.5 % sodium hypochlorite) for 20 min. The seeds were rinsed with sterile, deionized water four times (sequentially for 30s, 5 min, 10 min and 15 min), next left in 4°C for 19-20h to imbibe. Seeds were once again surface-sterilized using 70% ethanol and 10% $HgCl_2$ and rinsed with sterile water. Then embryos were excised and cultured on nutrient media.

Culture media, induction and regeneration : Mature embryos from the endosperm in imbibed seeds were separated in aseptic conditions and scutellum side up with plumule slightly embedded in the callus induction media in 90 mm Petri dishes were placed. Explants were cultured on MS (Murashige and Skoog, 1962) standard medium consisting of MS micro- and macro-nutrients, MS vitamins and solidified with 0.3 % Gellan Gum (Gelrite®, Sigma Aldrich). In study, the effect of three types of auxins: 2,4 dichlorophenoxyacetic acid (2,4-D); 3,6-dichloro-o-anisic acid (dicamba); 1-naphthaleneacetic acid (NAA)] and various sources of carbohydrates (Table 2.) on callus induction were tested.

Table 2. Components of MS medium modification

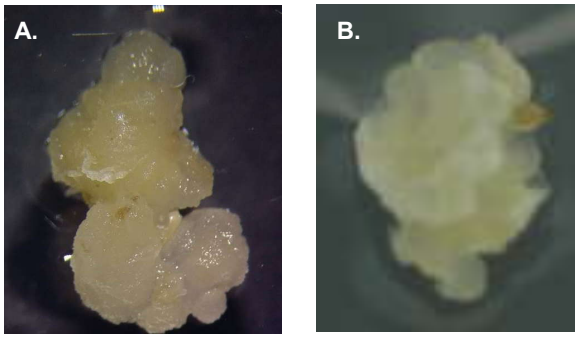
	MEDIUM TESTED							
Components	**S**	**M**	**S 2,4-D**	**M2,4-D**	**S NAA**	**M NAA**	**S D**	**M D**
2,4-D	-	-	+	+	-	-	-	-
NAA	-	-	-	-	+	+	-	-
dicamba	-	-	-	-	-	-	+	+

The medium components were dissolved and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Petri dishes containing fifteen cultured embryos were sealed with Parafilm and incubated under dark conditions at 25°C±1°C for 4 weeks. All calluses produced by the embryos were transferred to MS medium with half strength macronutrients, without growth hormones and cultured at 25 ± 1°C under 16-h photoperiod. After 2 week interval subcultures were performed. When roots and shoots were established, young plants were transferred to a flask with the same medium and cultured under the same conditions. The percentage of embryos producing an embryogenic callus, non-embryogenic callus and plant regenerating embryo ratios were used to assess phenotypic response of wheat genotypes in the *in vitro* culture.

Results and discussion

Embryogenic callus induction : In general, two types of calluses in the study were observed: embryogenic (EC) and non-embryogenic callus (NEC) (Figure 1.). EC was compact, nodular, creamy white colour and contained embryonic structures occurring either as independent or fused nodules, while NEC was characterized by a soft, loose, friable nature and cream colour.

Figure 1. A. Embriogenic calluc, B. Non-embriogenic callus



After two weeks on the induction medium, embryos were generating embryogenic callus. There was a higher percentage of EC in many treatments, consistent with previous observations made on calluses induced from wheat mature embryo cultures (MacKinnon et al. 1987).

Effects of auxins and sugar on callus induction

The type of auxins and carbon sources played an important role in embryogenic callus induction. All winter wheat cultivars used in this study enhanced callus induction on all tested media (Table 3). The highest percentage of embryos producing EC was obtained for cultivar Ostroga, while the smallest one for cultivar Bamberka (Figure 2). The rate of EC formation on media supplemented with dicamba was generally better than on media with NAA and 2,4-D. A significant difference was observed for Bamberka. The percentage of embryogenic callus on dicamba containing medium was 69.9% when for 2,4-D 52.4% and for NAA barely 47.1%. The type of sugar used in the media did not have any effect on the production of callus from mature embryos – the differences ranged from 1.4% for cv. Wydma to 5.1% for cv. Arkadia (Figure 3).

Table 3. Effect of 2,4-D, NAA and Dicamba on embryogenic callus formation.

Cultivars	**Auxin**	**Callus induction [%]**	**Embriogenic callus induction**	
			Maltose	**Sucrose**
AKRADIA	control	100.0	92.1	57.1
	2,4-D	97.7	58.1	78.9
	NAA	75.3	87.8	96.7
	Dicamba	86.4	90.4	90.1
ASTORIA	control	95.7	57.3	71.5
	2,4-D	82.4	59.5	60.8
	NAA	71.6	60.7	83.3

	Dicamba	92.1	77.2	80.47
BAMBERKA	Control	79.9	45.5	32.3
	2,4-D	32.8	95.8	33.8
	NAA	41.8	21.1	66.7
	Dicamba	94.7	71.2	68.1
MUZA	control	96.8	91.14	58.02
	2,4-D	86.0	66.79	94.4
	NAA	94.0	83.1	96.4
	Dicamba	93.6	77.1	81.9
OSTROGA	control	99.1	91.0	94.0
	2,4-D	100.0	99.4	97.5
	NAA	94.1	94.1	97.3
	Dicamba	92.7	100.0	100.0
WYDMA	control	93.7	78.3	80.0
	2,4-D	84.1	58.9	65.34
	NAA	83.0	70.2	66.6
	Dicamba	93.3	87.4	80.0

It was found, that the embryogenic callus induction among the wheat cultivars tested *in vitro* culture by using as explants mature embryos was regulated by the type of the auxin and carbon source used to supplement the media. A lot of workers have successfully used high concentration of 2,4-D for callus formation. However, 2,4-D auxin used at higher doses was increasing chromosomal instability and somaclonal variation (Ziauddin and Kasha; 1990). Picloram and dicamba have been used as alternatives (Satyavathi et al.; 2004). Mendoza and Kaeppler (2002) were demonstrated, that medium supplemented with dicamba was the most effective in promoting callus production from mature embryos of 'Bobwhite'[11]. According to Filippov et al. (2006) dicamba was more effective to induce embryogenic callus from mature embryos than 2,4-D or 2,4-5-T. In our study 38.3% of embryos generated EC on medium with dicamba, while it was only 13.1% for medium modified by 2,4-D at the same concentration of the growth hormones. As opposed to our study Ren et al., (2010) reported a significant effect of sugar on the production of callus from mature embryos. In all cultivars, replacement of sucrose by maltose increased the mean percentage of callus production from 76.6 to 77.8%. Our data suggest, that the addition of dicamba in induction culture medium was essential for embryogenic callus formation, however, it was influenced by wheat genotype.

Figure 2. Effect of phytohormones on embryogenic callus induction

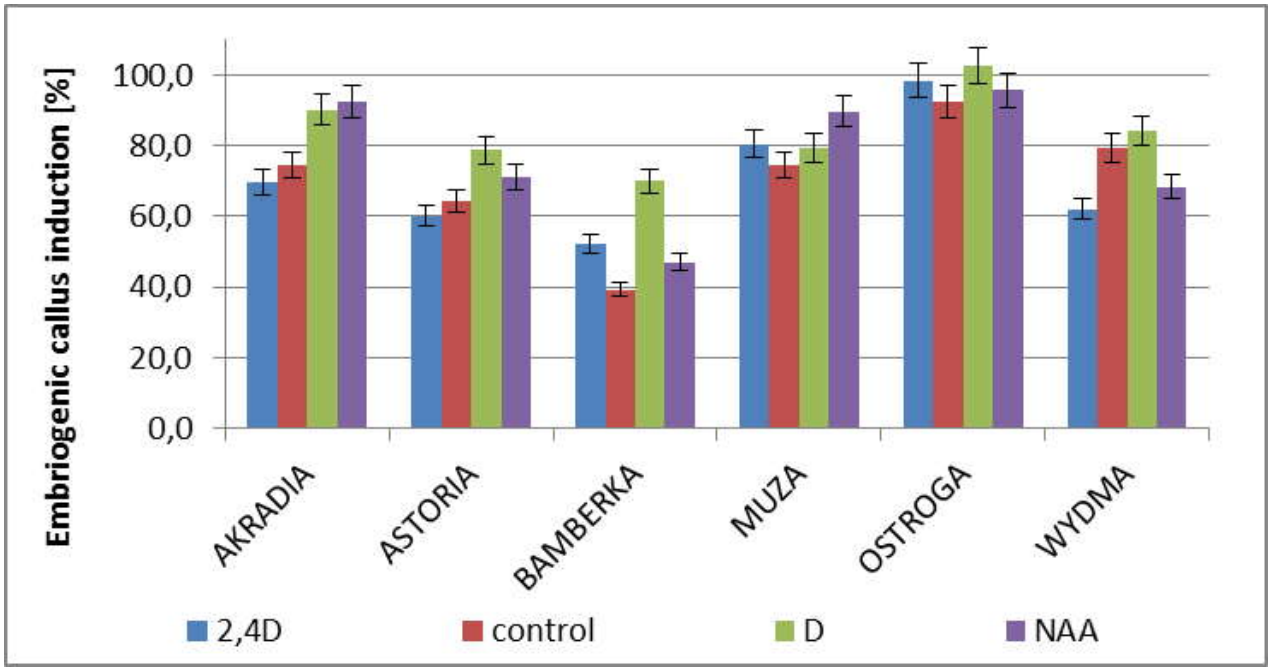
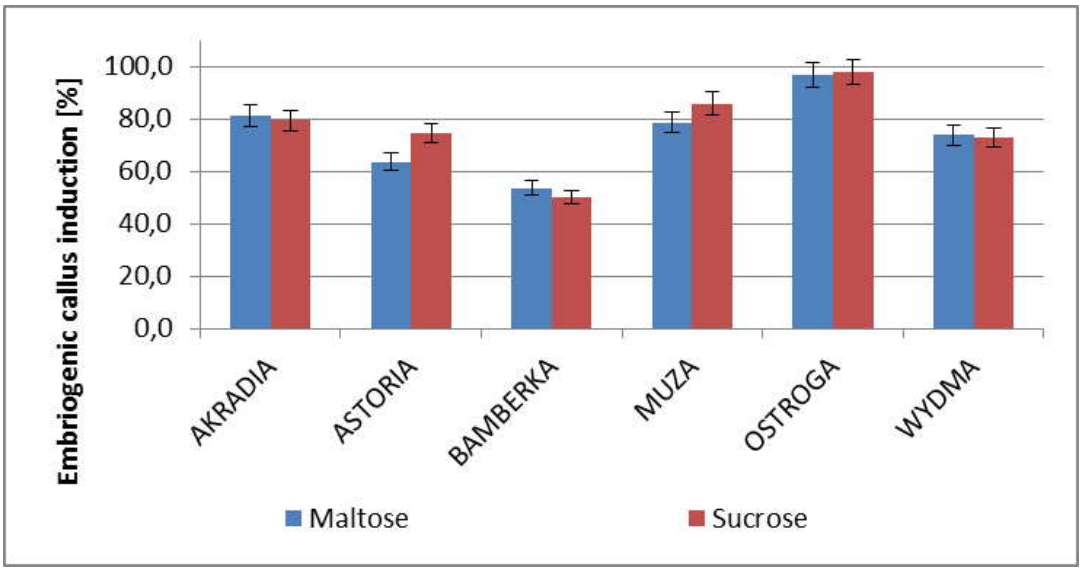


Figure 3. Effect of carbon source on embryogenic callus induction



Effects of auxins and sugar on plant regeneration in vitro culture

After 4 weeks culture incubation in darkness, the callus on regeneration media for shoot differentiation was placed. The efficiency on plant regeneration was assessed by counting the number of callus producing seedlings. Table 4 presents the results for plant regeneration and these indicated that there was a significant difference in plant regeneration capacity between the different treatments used in the inducing medium.

Table 4. Effect of 2,4-D, NAA and Dicamba on plant regeneration.

CULTIVARS	Auxin	No. of plantlets regenerated per embryogenic callus[%]	
		Maltose	Sucrose
AKRADIA	control	8.2	0.0
	2,4-D	0.0	0.0
	NAA	1.4	0.0
	Dicamba	0.0	3.8
ASTORIA	control	0.0	9.1
	2,4-D	0.0	0.0
	NAA	2.3	8.0
	Dicamba	0.0	7.0
BAMBERKA	Control	0.0	0.0
	2,4-D	0.0	0.0
	NAA	0.0	0.0
	Dicamba	1.8	0.0
MUZA	control	0.0	13.0
	2,4-D	0.0	0.0
	NAA	18.7	1.2
	Dicamba	0.0	0.0
OSTROGA	control	5.0	27.7
	2,4-D	2.4	0.0
	NAA	15.2	9.8
	Dicamba	17.3	12.1
WYDMA	control	7.1	0.0
	2,4-D	0.0	0.0
	NAA	2.9	0.0
	Dicamba	0.0	20.3

All of wheat cultivars showed *in vitro* plant regeneration capacity. The greatest rate of plant regeneration was observed for cv. Ostroga and the lowest one for cv. Bamberka. Media without growth hormones had a significant effect on the number of plantlets regenerated per EC. The largest difference was observed for cv. Ostroga. The percentage of plants regenerated on control medium was 16.3%, while for 2,4-D it barely reached 1.2% (Figure 4). Efficiency of plant regeneration was not strongly influenced by the sugar type used in media (Figure 5).

Figure 4. Effect of phytohormones on plant regeneration.

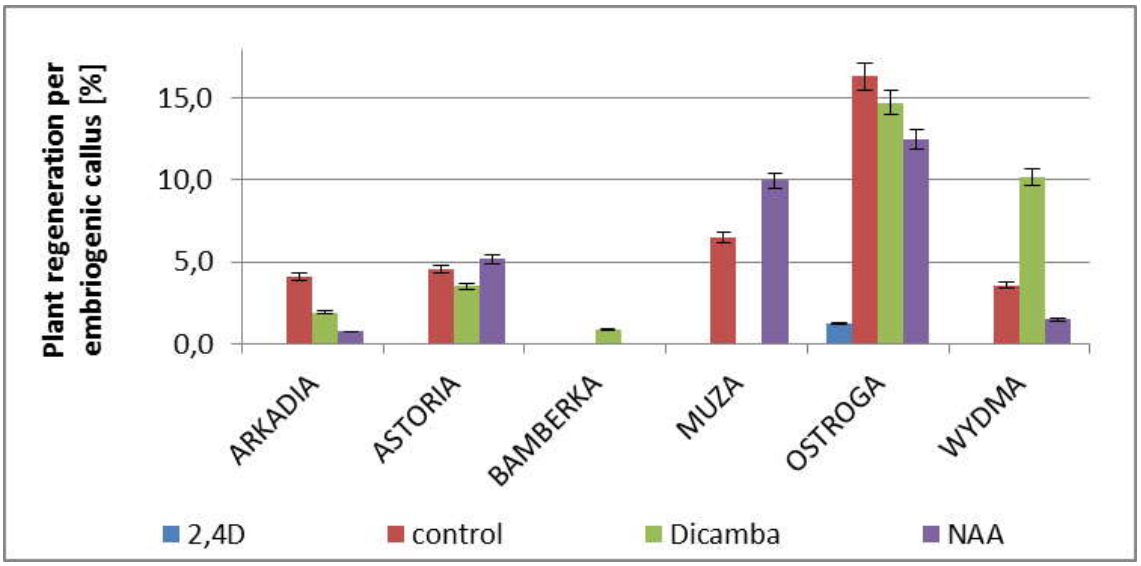
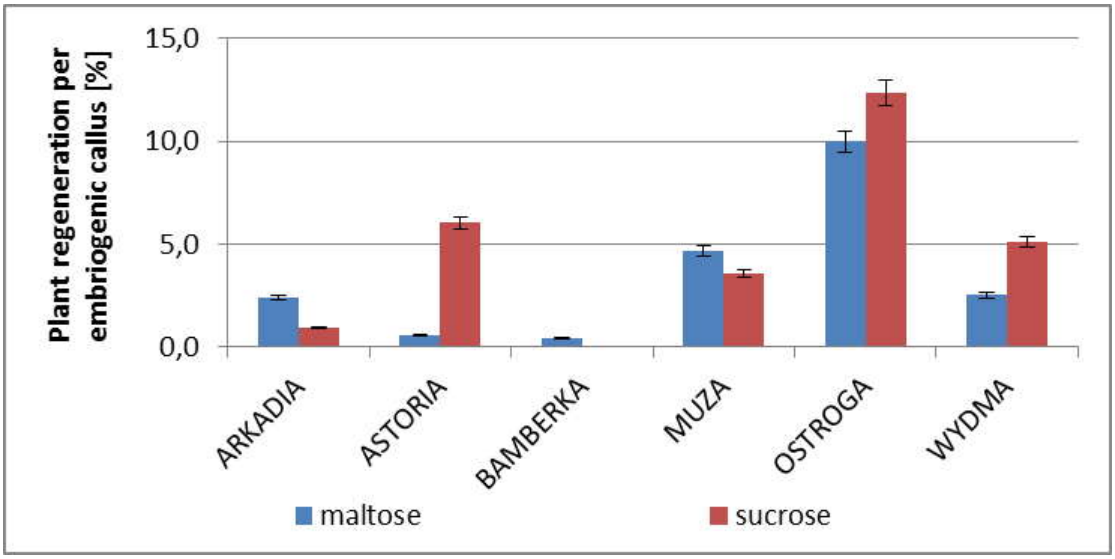


Figure 5. Effect of carbon sources on plant regeneration.



Researches still improve plant regeneration efficiency from mature embryos of wheat. Generation of callus is regulated by the type of the auxin and sugar source used in culture medium. Many studies showed that dicamba influences plant regeneration capacity. Przetakiewicz et al. (2003) noted the highest plantlets number (37 plantlets) for cv. Torka, whose embryos were previously cultured on dicamba. In report of Papenfuss and Carman (1987) dicamba influence on regeneration capacity of wheat was substantially larger than 2,4-D. Also, according to Ozgen et al. (1998) the highest regeneration rates (8.5 plants per embryo) among winter wheat genotypes were obtained from specific treatments using 18mM dicamba and sucrose. In general, our study documents that modified medium containing maltose, without auxins had a significant effect on the number of plantlets regenerated.

In conclusion, we have demonstrated significant effects of growth hormone type and their interaction on embryogenic callus formation and plant regeneration from cultures initiated from mature embryos of wheat genotypes. Inducing media with dicamba played an important role in embryogenic

callus induction, however, the highest efficiency of plant regeneration was obtained on medium without auxins. In our study, we have not observed a significant role of sugar type added to the media we used. The results of our study may add to future practical application of wheat mature embryo culture for breeding, transformation and other biotechnological objectives.

Acknowledgements

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