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Effect of Methyl Jasmonate on Carbohydrate Composition, α-Amylase Activity and Growth of Triticale (*Triticosecale* Witmmack) Seedlings

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ABSTRACT

The effect of Methyl Jasmonate (MJ, jasmonic acid methyl ether) at 10^{-6}-10^{-3}M concentrations on triticale kernels germination, seedling growth, changes in soluble carbohydrates content and composition, and activity of α-amylase was studied. MJ inhibited the germination of triticale kernels, possibly due to decreasing activity of α-amylase, leading to the depletion of soluble carbohydrates in both embryonic and endosperm tissues. In this way, MJ reduced starch degradation. A lower amount of soluble carbohydrates in germinating seeds decreased water uptake (between 24 and 72 hours of germination) and delayed seedling development. The above effect can be attributed to high concentrations of MJ in the incubation mixture (10^{-3}M, 10^{-4}M). MJ reduced the number of embryonic roots in 5-days-old seedlings in all examined concentration ranges.

Keywords: Alpha-amylase, Carbohydrates, Germination, Methyl jasmonate, Triticale.

INTRODUCTION

A sound knowledge of the mechanisms underlying the control and hormonal regulation of seed germination and seedling growth is critical for improving crop establishment and yield. Seed germination is partly controlled by endogenous phytohormones. Abscisic Acid (ABA) and Gibberellins (GAs) have an antagonistic relationship. ABA establishes and maintains seed dormancy, whereas GAs stimulate germination. GAs and ABA antagonistically regulate downstream mechanisms that mediate two key processes during the completion of endospermic seed germination: embryo elongation and endosperm weakening (Linkies and Leubner-Metzger, 2012). Both phytohormones regulate the expression of genes encoding the enzymes required for protein storage (Cercós *et al.*, 1999) and carbohydrate mobilization (Appleford and Lenton, 1997). During the germination of cereal kernels, α-amylases play a key role in the mobilization of energy reserves stored in insoluble starch granules. Genes encoding α -amylases are gibberellin-inducible; their expression begins in the scutellar epithelium and continues in the aleurone layer (Sugimoto *et al.*, 1998). In germinating wheat kernels, glucose released from degraded endosperm starch is converted to sucrose in the scutellum (Aoki *et al.*, 2006).

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During early germination, sucrose is the major sugar in the endosperm, whereas maltose and glucose are the predominant sugars in successive stages of development (Aoki *et al*., 2006; Scofield *et al*., 2007). The activity of α-amylase can be inhibited by Methyl Jasmonate (MJ), and the above was observed in the germinating seeds of *Amaranthus caudatus* (Białecka and Kępczyński, 2003a), *Amaranthus hypochondriacus* (Dèlano-Frier *et al*., 2004) and maize (Norastehnia *et al.*, 2007). Jasmonates (JAs) are known to inhibit seed germination (Linkies and Leubner-Metzger, 2012), but their effect on carbohydrate metabolism in germinating cereals has not been explained to date. In germinating lupine seeds, MJ inhibits the hydrolysis of Raffinose Family Oligosaccharides (RFOs) and galactosyl cyclitols, the first reserves for germinating embryos (Zalewski *et al*., 2010). The above can be attributed to the inhibition of one of the three forms of □-d-galactosidase. In cereal kernels, RFOs are present in small amounts (Barnes, 1982), therefore, their storage functions seem doubtful. In the present study, the effect of MJ on seed germination, seedling growth, soluble carbohydrate composition and α-amylase activity in winter triticale kernels were investigated.

MATERIALS AND METHODS

Plants

The experimental material comprised winter triticale (*Triticosecale* Witmmack cv. Moderato) kernels harvested at the full ripe stage in experimental plots of the University of Warmia and Mazury in Olsztyn. The kernels were stored in a laboratory for 6 weeks (until the end of after-harvest ripening), in linen bags, at a temperature of 20-25^{o}C and relative air humidity of 60-65%. Germination capacity was evaluated after 8 days of kernel incubation (at 20°C in darkness) on Petri dishes lined with the wet germination paper (Anchor Paper Company, St. Paul, USA). The effect of various concentrations of MJ (Sigma USA) on kernel germination and seedling growth was determined after 24, 72 and 120 hours of kernel incubation in the darkness (at 20°C). MJ was initially dissolved in 1 mL of 99% ethanol, and then diluted with a sterile double distilled water to obtain 1 L of MJ at 10^{-3}M concentration. This stock solution was diluted with the water to obtain MJ solutions at 10^{-4}, 10^{-5} and 10^{-6}M concentration. Kernels incubated in distilled water with 0.1% ethanol were the control. Kernels were incubated in control and MJ solutions in four replicates of 100 kernels each.

Microscopic Analysis

After 120 hours of incubation, whole kernels were fixed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer with pH 7.3 at 4°C to prepare samples for analysis under an optical microscope. The examined material was washed and dehydrated in ethanol. Kernels intended for evaluation under an optical microscope were cut into slices, stained with I_2/KI diluted with water (1:2, v/v) and examined under a Nikon Eclipse 80i microscope.

Extraction and Analysis of Soluble Carbohydrates

Soluble carbohydrates were extracted according to a previously described method (Lahuta and Goszczyńska, 2010). Briefly, the embryos of dry and germinating triticale kernels were separated from the endosperm, weighed, frozen in liquid nitrogen and lyophilized. Dry tissues were crushed in a mixed mill (MM200, Germany). Carbohydrates were extracted from 10-20 mg of ground embryonic tissue or 40 mg of endosperm tissue 400-800 µl of 50% ethanol, containing 100 µg of xylitol, as an internal standard. Samples were vigorously mixed and incubated at 90ºC for 30 minutes. After cooling to room temperature, samples

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were centrifuged (21,000×g, 30 minutes) and clear aliquots were deionized (with a mixture of cationic and anionic ion resins, both DOWEX type, SIGMA, USA). After centrifugation, 100-200 µl of aliquot was dried in a speed-vacuum rotary evaporator to dryness. Dry residues were derivatized with 200 µl of the TMSI (trimethylsilyl imidazole): pyridine mixture (1:1, v/v) at 80ºC for 45 minutes. TMS-derivatives of carbohydrates were separated GC on a ZEBRON ZB-1 capillary column (Phenomenex, USA) according to a previously described method (Lahuta, 2006). Carbohydrate standards were purchased from Sigma (USA). Carbohydrate content was calculated from standard curves of the appropriate components. The amounts of unknown carbohydrates with Retention times (Rt) of 6.52 and 7.39 were calculated based on the nearest known standards (1-kestose and stachyose, respectively).

Determination of α-Amylase Activity

The activity of α-amylase was assayed in kernels germinating for 72 hours at 20ºC in the presence of MJ at concentrations of 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M. Using a mortar and pestle, soluble proteins were extracted from embryos (100) and endosperm (20) with 1 ml of 0.2M NaCl in 0.01M phosphate buffer (pH 7.0). After centrifugation (16,000×*g*, 4°C, 20 minutes), protein concentration in the homogenate was assayed with the use of the Bradford reagent (Bio-Rad). Enzyme activity was assayed according to the method proposed by Sitarski *et al*., (1992). The extract was incubated with 1% insoluble starch solution (in 0.1M phosphate buffer, pH 5.0) at 37°C for 5 minutes. After addition of 0.5% J_2 in 1% KJ (in 0.1N HCl) solution, the absorbance was read at the wavelength of 620 nm. A change in absorbance (at the wavelength of 620 nm) by 0.1 min^{-1} g^{-1} DW of tissue was adopted as the Unit of enzyme activity (U). The results were presented as the means of three replicates.

Statistical Analysis

The results were presented as the means of three (carbohydrates and enzyme activity) or four (germination) independent replicates. The results were processed statistically by ANOVA and Tukey's post-hoc test.

RESULTS AND DISCUSSION

The lowest concentrations of exogenous MJ (10^{-6}M) applied during germination of triticale kernels are similar to the natural amounts of endogenous jasmonates that occur in plants and seeds. (Saniewski and Czapski, 1999). Jasmonates are present in the seeds of many plants, and their concentrations range from 10 to 100 ng g^{-1} FW, subject to species (Białecka and Kępczyński, 2003). In other studies, JA concentrations in 6-days-old maize seedlings infested by *Spodoptera exigua* larvae increased to 72-75 ng g^{-1} FW, and they were determined at 0.7-10.4 ng g^{-1} FW in control plants (Schmalz *et al*., 2003). In our study, the concentration of MJ (both endogenous and after treatment) in triticale kernels was not analyzed. However, results clearly indicated the inhibitory effect of MJ at increasing concentration in incubation medium on kernels germination and seedling growth (Table 1). The inhibitoriest effect on germination and seedling growth was observed in caryopses treated with 10^{-3}M MJ (Table 1). High MJ concentrations (10^{-5} to 10^{-3}M) had an adverse impact on grain germination, which was manifested by reduced germination capacity and lower increase in fresh weight and dry weight of seedlings after 72 and 120 hours of incubation. The negative effect of the analyzed hormone on germination capacity was directly proportional to its concentrations (Table 1). It should be noted that all tested concentrations of MJ significantly decreased the number of rootlets in germinating kernels (Table 1). The negative effect of the highest MJ concentration on water uptake by embryo and seedlings was eliminated after 120 hours of germination. In other studies, JA has been

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Table 1. The effect of MJ on triticale kernels Dry Weight (DW), Fresh Weight (FW), Water Content (WC) and germination (after 72 and 120 hours in %), in grains and seedlings during 120 hours of germination at 20°C (in the dark).

Treatment	24h				72h				120h				
	DW of embryo (mg)	FW of embryo (mg)	WC in embryo (%)	DW of seedling (mg)	FW of seedling (mg)	WC in seedlings (%)	Germination capacity (%)	DW of seedlings (mg)	FW of seedling (mg)	WC in seedlings (%)	Number of embryonic roots	Germination capacity (%)
Control	1.41[a,α]	5.51[a]	74.41[a]	5.23[a]	36.5[a]	85.67[a]	84.25[a]	11.00[a]	76.5[a]	85.62[a]	5.00[a]	86.25[a]
MJ 10^{-6}	1.39[a]	5.20[a]	73.27[a]	5.11[a]	33.7[a]	84.84[a]	83.50[a]	10.20[ab]	70.6[a]	85.55[a]	4.05[b]	87.00[a]
MJ 10^{-5}	1.37[a]	5.32[a]	74.25[a]	4.09[a]	18.3[b]	77.65[a]	70.00[c]	8.70[ab]	61.2[b]	85.78[a]	4.15[b]	73.50[c]
MJ 10^{-4}	1.31[a]	4.28[b]	69.39[ab]	3.62[c]	15.7[b]	76.94[a]	75.50[b]	7.70[ab]	54.5[b]	85.87[a]	4.01[b]	79.00[b]
MJ 10^{-3}	1.32[a]	3.85[b]	65.71[b]	2.10[c]	4.6[c]	54.35[b]	57.00[d]	6.70[b]	41.4[c]	83.82[a]	3.93[b]	62.50[d]
LSD	1.21	3.13	4.39	0.51	2.36	6.71	3.46	2.03	5.53	2.69	0.38	3.46
P= 0.01												

[α] Values (means of four replicates) with the same letter are not significantly different (P< 0.01).

found to inhibit stem elongation in *Nicotiana attenuata* plants *via* inhibition of GA biosynthesis in stems (Heinrich *et al*., 2013). Moreover, wound-induced jasmonates stunted the growth of Arabidopsis plants through the suppression of mitosis (Zhang and Turner, 2008).

Changes in Soluble Carbohydrates

Sucrose was the major soluble carbohydrate in the embryos of dry kernels (189.39 mg g^{-1} DW, 85% of Total Soluble Carbohydrates, TSC). The embryo also contained monosaccharides (fructose and glucose), raffinose, 1-kestose, *myo*-inositol, sorbitol and several unidentified compounds [presumably carbohydrates with a Degree of Polymerization (DP) of 3 and 4], which disappeared during grain germination (data not shown). Only one compound (DP4) continued to be present throughout germination. The carbohydrate composition of embryonic and endosperm tissues was somewhat different (Table 2). Sorbitol, raffinose and DP4 were not observed in the endosperm of dry caryopses, and maltose was the predominant soluble carbohydrate (15.79 mg g^{-1} DW, 49% of TSC). Maltose was not detected in the embryo. Carbohydrate concentrations were seven-fold higher in the embryo than in the endosperm.

For comparison of changes in the content of soluble carbohydrates during kernels germination, the concentrations of sugars in embryo and endosperm of dry seeds (Table 1) were expressed in μg $embryo^{-1}$ or μg $endosperm^{-1}$ (Figures 1-3). The TSC content of the embryo of control caryopses (treated with MJ at concentration 0M, control) decreased two-fold during the first 24 hours of germination (Figure 1-A). During that period, sorbitol and raffinose disappeared completely from the embryos (data not shown), and sucrose levels decreased approximately three-fold (Figure 2-C). Embryo tissues contained elevated amounts of 1-kestose (Figure 2-D) and an unknown

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Table 2. The concentration of soluble carbohydrates in embryo and endosperm of dry mature kernel of triticale.

Carbohydrate	Embryo	Endosperm
	mg g^{-1} DW	
Fructose	3.81 ± 0.31	0.08 ± 0.00
Glucose	2.69 ± 0.07	0.62 ± 0.04
Sorbitol	7.24 ± 0.16	0
Myo-inositol	0.90 ±0.02	0.04 ± 0.01
Sucrose	180.39 ± 5.31	9.99 ± 0.31
Maltose	0	15.79 ± 2.07
Raffinose	6.65 ± 0.12	0
1-Kestose	7.60 ± 0.26	5.25 ± 0.27
UNK (DP3)	0	0.13 ± 0.05
UNK (DP4)	2.95 ± 0.11	0
Total Soluble Carbohydrates (TCS)	221.23 ± 6.28	32.3 ± 2.54

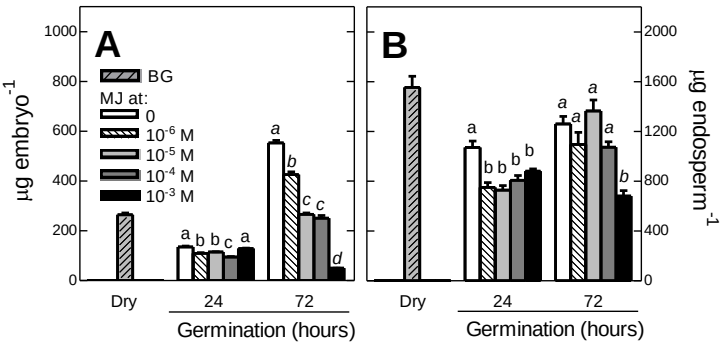

Figure 1. The content of total soluble carbohydrates in embryo (A) and endosperm (B) of triticale kernel before germination (Dry, BG) and after germination for 24 and 72 hours at 20ºC in the presence of MJ at 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M concentration. Means of three replicates±SE. Bars with the same letters are not significantly different ($P< 0.05$) after one way ANOVA and Tukey's correction.

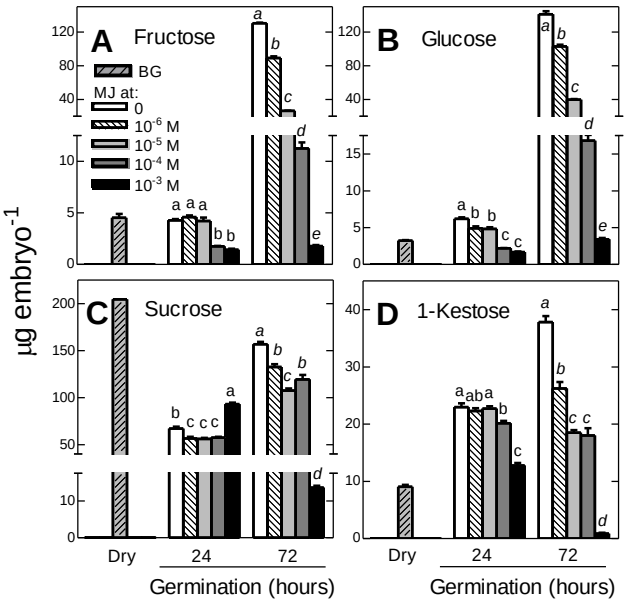

Figure 2. The content of fructose (A), glucose (B), sucrose (C) and 1-kestose (D) in embryo of triticale kernel before germination (Dry, BG) and after germination for 24 and 72 hours at 20ºC in the presence of MJ at 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M concentration.

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compound (DP4, 8.75±0.19 µg), whereas a minor increase was noted in fructose and glucose levels (Figures 2-A and -B). With the progress of germination, monosaccharides, sucrose and 1-kestose concentrations increased in the tissues of growing coleoptiles and in the roots (Figures 2A-D, white bars). The glucose content of the endosperm of control caryopses increased with germination (Figures 3-A and -B). A reverse trend was noted for sucrose and 1-kestose (Figures 3-C and -D). A roughly two-fold decrease in maltose levels in the endosperm of dry caryopses in the first 24 hours of germination was followed by an increase in maltose concentrations (Figure 3-B).

The dramatic decrease in the sucrose (Figure 2-C) and raffinose (data not shown) content of control embryos during the first 24 hours of triticale grain germination seems to confirm that both carbohydrates serve as primary reserve materials. In legumes, delayed germination could be attributed to inhibited breakdown of RFO (Blöchl *et al*., 2007; Lahuta and Goszczyńska, 2010; Zalewski *et al*. 2010). However, raffinose was present in the embryos of triticale kernels at low concentrations (6.65±0.12 mg g^{-1} DW, Table 2) and its role as reserve material in triticale kernels cannot be compared with legume seeds where RFO concentrations in the embryonic axis ranged from several to 20% of dry weight (Górecki *et al*., 2001). Decreased levels of maltose (Figure 3-B) and 1-kestose (Figure 3-D) in endosperm of triticale kernels also suggest that similar to sucrose, both sugars are sources of energy before the starch broken down in the endosperm becomes the main source of carbohydrates. Interestingly, the degradation of sucrose, raffinose, starch and fructans was not correlated with monosaccharide, glucose and fructose levels in the embryo (Figures 2-A and -B), which indicates that the released monosaccharides are rapidly utilized in tissue metabolism. During the successive 48 hours of germination, monosaccharide concentrations in shoot and root tissues of developing seedlings increased rapidly, and similar observations were made in germinating wheat (Aoki *et al*., 2006), rice (Scofield *et al*., 2007) and barley (Sreenivasulu *et al*.,

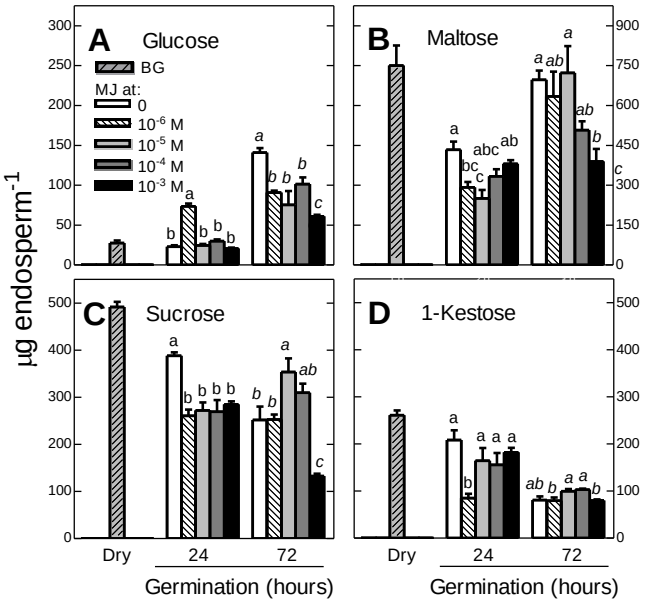

Figure 3. The content of glucose (A), maltose (B), sucrose (C) and 1-kestose (D) in endosperm of triticale kernel before germination (Dry, BG) and after germination for 24 and 72 h at 20ºC in the presence of MJ at 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M concentration.

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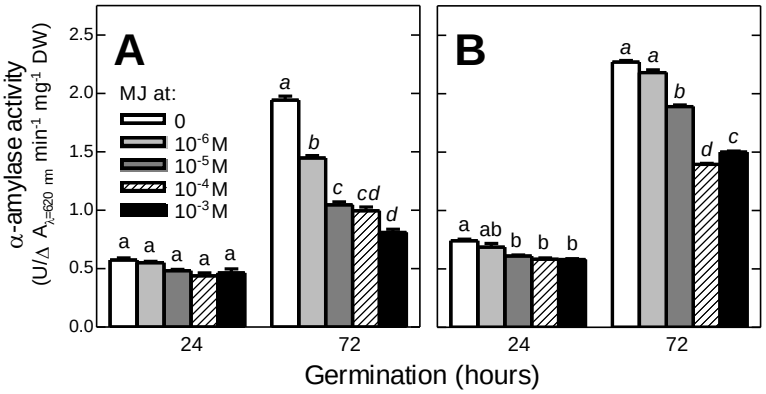

Figure 4. The activity of α-amylase in embryo (A) and endosperm (B) of triticale kernel germinating for 72 hours at 20ºC in the presence of MJ at 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M concentration.

2008; Frank *et al*., 2011). The reported increase in TSC (about 400 µg per seedling, Figure 1-A) can be attributed to the release of energy reserves from endosperm tissues. The increase in sucrose and monosaccharide levels could result from the conversion of glucose into sucrose in the scutellum, and a similar increase was noted in germinating wheat kernels (Aoki *et al*., 2006). However, the variations in monosaccharide and sucrose concentrations between 24 and 72 hours of germination point to progressive degradation of other storage materials, such as starch, in both embryonic and endosperm tissues. The increased activity of α-amylase (Figure 4) and degradation of starch (Figure 5) seems to be a confirmation of this hypothesis.

The content of soluble carbohydrates in the embryos and endosperm of germinating triticale kernels was significantly affected by MJ, in MJ concentration and germination duration-dependent manner. During the first 24 hours of germination the content of monosaccharides decreased in embryos of caryopses treated with MJ at 10^{-4} and 10^{-3} M concentrations (Figures 2-A and -B). The level of sucrose slightly decreased, except for embryos of caryopses treated with MJ at highest concentration (Figure 2-C), whereas the accumulation of 1-kestose was slowed by MJ (at 10^{-4} and 10^{-3}M, Figure 2-D). At the same time, the levels of maltose, sucrose and 1-kestose in the endosperm were lower in kernels germinating in MJ (regardless of MJ concentration) than in control (Figures 3B-D).

Between 24 and 72 hours of germination, a significantly smaller increase in monosaccharide and sucrose concentrations was noted in embryonic tissues of MJ-treated kernels (Figure 2A-C), which indicates that MJ disrupted carbohydrate supply from the endosperm. This depletion of sucrose, maltose and monosaccharides (Figures 2 and 3) can be a result of reduced starch degradation (Figure 5). In the endosperm of control caryopses, starch granules were completely degraded after 120 hours of germination, but they were still present in the endosperm of caryopses treated with MJ (Figure 5). In comparison with control, the highest amount of starch was observed in tissue sections treated with 10^{-3}M MJ. Only individual starch kernels were observed in caryopses treated with 10^{-6}M MJ (Figure 5-B – arrows). In embryonic and endosperm tissues, the presence of MJ decreased the activity of α-amylase (Figure 4), which inhibited starch degradation (Figure 5). As the result, lower levels of maltose and glucose were released from starch than in control caryopses (Figures 3-A and -B).

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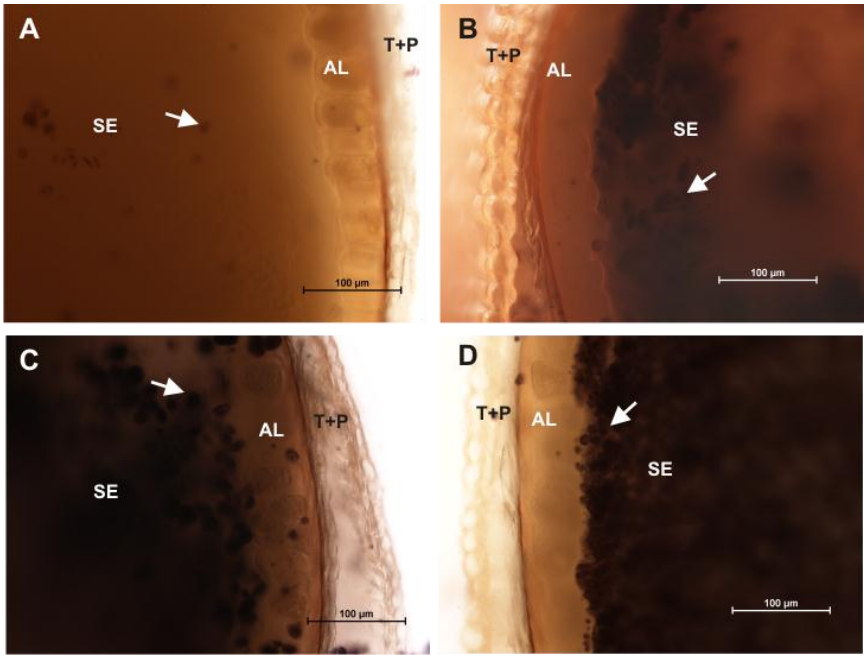

Figure 5. Tissue structure of triticale caryopsis after 120 hours of germination in water (A), 10^{-6} (B), 10^{-4} (C) and 10^{-3}M MJ (D), stained with diluted I_2/KI. T+P: Testa and Pericarp; AL: Endosperm Aleurone Layer, SE: Starchy Endosperm. Scale bar: 100 µm.

The germination of cereal kernels requires the activation of reserve-degrading enzymes and the weakening of the seed coat. Those processes are regulated through combined action of selected hormones (Barrero *et al*., 2009; Linkies and Leubner-Metzger, 2012). In our study, the activity of α-amylase in both embryonic and endosperm tissues of germinating triticale kernels was strongly inhibited by MJ (Figure 4), and MJ/JA exerted a similar effect on α-amylase activity in *Amaranthus caudatus* (Białecka and Kępczyński, 2003a) and *Amaranthus hypochondriacus* seeds (Dèlano-Frier *et al*., 2004). Although jasmonates' regulatory effect on the expression of selected genes has been well established (Memelink, 2009; Linkies and Leubner-Metzger, 2012), further work is needed to explain MJ's influence on the regulation of selected hydrolases. Białecka and Kępczyński (2003b) demonstrated that MJ's inhibiting effect on germination of *Amaranthus caudatus* seeds can be reversed by gibberellins. In another study, the cited authors observed that gibberellins and ethylene stimulated the degradation of RFOs and that seeds treated with MJ had a higher maltose and maltotriose content (Białecka and Kępczyński, 2007). Therefore, it seems possible that the gibberellin-triggered expression of α-amylase in the scutellum and aleurone layer in the endosperm of cereal kernels (Sugimoto *et al*., 1998) is inhibited by MJ. Although there have been several reports of crosstalk between JA and GA signaling pathways, mostly documenting the antagonistic effect of GA on JA signaling (Yang *et al*., 2012), the antagonistic effect of MJ on the stimulation of α-amylase expression by GA's in triticale kernels was not studied. It can be suggested that inhibition of α-amylase activity by MJ is a result of MJ and GA interactions, which is a hypothesis needing further verification.

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اثر متیل جاسمونات در ترکیب کربوهیدرات، فعالیت α-آمیلاز و رشد نهال تریتیکاله (*Triticosecale* **Witmmack**)

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چکیده

اثر متیل جاسمونات (MJ، جاسمونیک اسید متیل اتر) در غلظت 10^{-3}-10^{-6} M بر جوانه زنی دانه های تریتیکاله ، رشد گیاهچه، تغییرات در محتوای کربوهیدرات محلول و ترکیب و فعالیت α-آمیلاز مورد مطالعه قرار گرفت. MJ باعث جوانه زنی دانه های تریتیکاله شده (احتمالا به خاطر افزایش فعالیت آلفا امیلاز) و منجر به کاهش کربوهیدرات محلول در بافت های جنینی و اندوسپرم می گردد. به این ترتیب، MJ تخمیر نشاسته را کاهش داد. مقدار کمی محلول کربوهیدرات ها در جوانه زنی بذر باعث

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کاهش جذب آب (بین ۲۴ تا ۷۲ ساعت جوانه زنی) و تاخیردر رشد شد. اثر فوق را میتوان به مربوط به غلظت بالایی ازMJ در مخلوط انکوباسیون (10^{-3}M, 10^{-4}M) نسبت داد. MJ تعداد ریشه های جنینی در نهال های ۵ روزه را در تمام طیف غلظت های آزمایش شده، کاهش داد.

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