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# Effects of daminozide on somatic embryogenesis from immature and mature embryos of wheat

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## Abstract

The influence of daminozide, an inhibitor of gibberellins biosynthesis, on the somatic embryogenesis in immature and mature embryo derived from tissue cultures of wheat was investigated. Low concentrations of daminozide (0.064-0.127 mM) positively affected the somatic embryogenic capacity of immature embryos (cv. 'Andros') cultured on the medium supplemented with either 2,4-dichlorophenoxyacetic acid (2,4-D) or dicamba or para chlorophenoxyacetic acid (*p*-CPA). The higher concentration of daminozide (0.51-1.02 mM) reduced the regeneration of shoots. The modification of exogenous/endogenous ratio of gibberellins by application of exogenous $GA_3$ did not remove the effect of daminozide. On the contrary, its combination with $GA_3$ inhibited the somatic embryogenesis and resulted in the supression of the development of somatic embryos. The supplement of 0.127 mM daminozide to 2,4-D containing medium increased the embryogenic competence of immature embryos of eleven Russian spring and winter varieties. For ten varieties the number of shoots produced per callus did not differ statistically from the control medium; one variety showed the increase of shoot number. In contrast with immature wheat embryos, mature ones (cv. Tajoznaja) did not response to daminozide supplementation either of the inductive or the different- tiative media. The present observations provide a method to increase the embryogenesis efficiency for wheat commercial varieties which immature embryos display low embryogenic potential.

***Abbereviations:*** 2,4-D_ 2,4-dichlorophenoxyacetic acid; *p*-CPA_ para chlorophenoxyacetic acid; $GA_{3\_}$ gibberellic acid; GAs _ gibberellins; ABA_ abscisic acid.

***Keywords***: *Triticum aestivum* L.; embryogenic callus; plant regeneration; inhibitor of gibberellins biosynthesis; gibberellic acid.

## Introduction

Somatic embryogenesis and efficient plant regeneration for spring and winter wheat varieties (*Triticum aestivum* L.) have been reported widely (Maddock et al. 1983, He at al. 1988; Bommineni and Jauhar 1996, Ozgen et al., 1996). In general, wheat callus induction and plant regeneration from tissue culture are influenced by culture medium, initial plant organ and, especially, by genotype (Maddock et al. 1983; He at al. 1988; Bommineni and Jauhar 1996, Özgen et al., 1998). Under the standard culture conditions, somatic embryogenesis from wheat tissue of the elite varieties was usually poor compared to the model varieties (Maddock et al. 1983; He at al. 1988; Fennell et al., 1996; Viertel et al., 1998). Such genotype variability

restricts the application of the modern biotechnologies like plant genetic transformation to a wider range of elite wheat varieties. Since the wheat transformation systems are based on the somatic embryogenesis from the competent cells, the transformation frequency is considered to be directly influenced by the peculiar ability of the initial explants. Genotype differences in the embryogenesis and plant regeneration are considered to be associated with variations in genetic programming response and the reprogramming of embryogenically competent cells by external factors (Tyankova and Zagorska 2001). To overcome these differences, particular environmental factors should be provided for tissue in such way that the physiological limitations could be partially compensated for readily available nutrient and hormone resources. A conventional approach is usually applied to improve the *in vitro* response of cereals: The explants of a given cultivar and various tissues are to be analyzed under different nutrient media. The importance of plant growth regulators has been demonstrated for a large number of cereals, especially the levels of synthetic auxins for callus induction and plant regeneration. Commonly 2,4-D considers to be the main phytohormone used for induction of somatic embryogenesis in wheat. Other substances with auxin-like activities such as dicamba and picloram, have been used as alternatives (Carman et al., 1987; Hunsinger and Schauz 1987; Barro et al., 1998). However several reports indicate that the combination of those substances with the other plant growth regulators is probably more promising in promotion of somatic embryogenesis from cereal tissues. Carman et all. (1987) showed that exogenous cytokinin (kinetin) in callus induction medium contained 2,4-D or dicamba, significantly increased the number of wheat embryoids. The addition of low abscisic acid (ABA) concentration to the culture medium has been reported to increase notably the frequency and extent of embryogenesis of spring wheat 'Chinese Spring' (Brown et al., 1989). The combination of dicamba and IAA increased the embryogenesis rate for wheat mature embryos (Filippov et al., 2006)

A little attention has been paid to the possible role of gibberellins (GAs) and inhibitors of gibberellins biosynthesis in somatic embryogenesis of wheat and other cereals. GAs are reported to accumulate in large quantities in the suspensor of the developing zygotic embryo and are, therefore, thought to play a key role during zygotic embryogenesis of cereals (White and Rivin 2000; Yang et al., 2003). However, it appears that for many species, the somatic embryogenesis is inhibited by the presence of exogenous gibberellins in the callus induction medium (Fujimura and Komamine 1975; Ezura and Harberd 1995; Gomes da Cunha and Ferreira 1996; Hutchinson et al., 1997). The inclusion of certain gibberellin biosynthesis inhibitors such as ancymidole, paclobutrazol or uniconazole influenced positively on the somatic embryogenesis and plant regeneration of several species (Feng and Wolyn 1993; Hutchinson et al., 1997; Senaratna et al., 2001; Chen and Chang 2003).
The objective of the present study was to investigate the influence of daminozide, the gibberellin biosynthesis inhibitor, on the efficiency of somatic embryogenesis and plant regeneration from immature and mature embryos of Russian wheat cultivars.

## Materials and methods

### *Plant material and explants preparation*

Wheat plants of spring and winter Russian wheat cultivars were grown in greenhouse with supplementary light to support 16h photoperiod and 23-25$^0$C day and 18-20$^0$C night temperature. Immature seeds were removed from the spikes 12-14 days post-anthesis and were surface sterilized with 70% ethanol for 2 min and 25% commercial bleach for 20 min followed by three rinses with sterile distilled water. Immature embryos were aseptically excised from the caryopses under a stereo microscope and placed scutellum up on a culture medium with the embryo-axis in a contact with the medium. Mature seeds were surface-sterilized with 96% ethanol for 1 min, treated for 15 min with 100% commercial bleach, containing a few drops of Tween 20 under constant stirring, and washed four times with sterile distilled water. The seeds were imbibed in sterile water for 3 h at room temperature with 3-4 water changes for more abundant extraction of sodium hypochlorite from seeds peel. Mature embryos were cut aseptically with scalpels without detaching from seeds as described Filippov et al., (2006).

### *Culture medium and conditions*

The basal Murashige and Skoog (1962) medium (MS) was used for all experiments. Before autoclaving at

***Table 1.*** Effect of daminozide and auxins on the efficiency of somatic embryogenesis and plant regeneration from immature embryos of spring wheat 'Andros'.

| Concentration of daminozide (mM) | Auxin type [a] | | | | | |
|---|---|---|---|---|---|---|
| | 2,4-D 9.05 μM | | Dicamba 9.05 μM | | *p*-CPA 1.69 μM | |
| | Somatic embryo-genesis (%) | Shoot number | Somatic embryo-genesis (%) | Shoot number | Somatic embryo-genesis (%) | Shoot number |
| 0.000 | 74 b | 12.9 a | 73 b | 11.1 a | 37 bc | 6.4 a |
| 0.064 | 87 a | 11.2 ab | 78 ab | 10.0 ab | 45 ab | 6.3 a |
| 0.127 | 91 a | 11.1 ab | 83 ab | 8.2 c | 55 a | 6.7 a |
| 0.255 | 90 a | 10.0 bc | 86 a | 6.0 d | 52 ab | 5.1 a |
| 0.510 | 86 a | 8.3 bc | 80 ab | 4.6 de | 49 ab | 4.0 ab |
| 0.765 | 85 a | 5.6 d | 64 c | 3.7 e | 35 bc | 2.0 bc |
| 1.019 | 60 c | 3.9 d | 59 c | 1.8 f | 22 c | 0.4 c |

a - Means within columns with the same letter are not significantly different at $\rho < 0.05$ according the Duncan's multiple range test

$121^0$C for 20 min pH was adjusted to 5.8. Plant-growth regulators solutions were filter sterilized and added to the autoclaved medium.

***Effect of daminozide on immature embryos***

In experiments spring cultivar 'Andros' was used. In preliminary trials it shown good callus induction and it was easier to grow 'Andros' plants, sterilize seeds and isolate uniform immature embryos in comparison with other genotypes. The culture medium for immature embryos contained the inorganic salts of MS medium supplemented with 150 mg $l^{-1}$ L-asparagine, 0.5 mg $l^{-1}$ thiamin, 30 g $l^{-1}$ sucrose and 7 g $l^{-1}$ Difco agar. Isolated immature embryos were placed into Petri dishes on callus induction medium supplemented with plant growth regulators and were incubated at 24-$26^0$C in the dark. After 30 days of incubation the developed calli with somatic embryos were transferred onto hormone-free MS medium for regeneration and were incubated in glass jars for 30 days at 24-$26^0$C at 16 h/8 h light/dark cycle provided by white and fluora tubes (1500 lux). Daminozide solution was added to the callus induction medium in order to give varying final concentrations (0, 0.064, 0.127, 0.255, 0.510, 0.765 and 1.019 mM) in combination with one of the following substances: 2,4-dichlorophenoxyacetic acid (2,4-D) (9.05 μM), 3,6-dichloro-*o*-anisic acid (dicamba) (9.05 μM), or *p*-chlorophenoxyacetic acid (CPA) (1.69 μM). Concentrations of auxin-like substances were chosen according to pilot studies which previously had been carried out in our laboratory (data is not shown). Immature embryos were cultured on callus induction medium supplemented with or without gibberelic acid ($GA_3$)(0, 0.29, 0.75, 1.44, 2.89 or 5.79 μM), or daminozide (0, 0.255 or 0.510 mM) and 9.05 μM 2,4-D for studying of the interaction of exogenously applied $GA_3$ and daminozide on wheat embryogenesis and regeneration. Immature embryos of eleven Russian wheat spring and winter cultivars were cultured on induction medium supplemented with 2,4-D (9.05 μM) either alone or in combination with daminozide (0.127 mM), to evaluate the effect of daminozide on the genotypic efficiency of somatic embryogenesis.

***Effect of daminozide on mature seeds/embryos***

A two-step protocol for somatic embryogenesis, which allows the separation of induction and differentiation phases, was used in experiments (Filippov et al., 2006). Mature seeds of 'Tajoznaja' were placed furrow downwards into Petri dishes on callus induction MS medium supplemented with 20 g/l sucrose, MS vitamins, 2,4-D (45.25 μM), indoleacetic acid (IAA) (2.85 μM) and daminozide (0, 0.127, 0.255, 0.510, 1.019 μM) and solidified with 7 g $l^{-1}$ Difco agar. 21 days after incubation at 24-26 $^0$C in the dark, the developed calli were detached from the seeds and placed into Petri dishes onto the same medium without auxines and daminozide for embryo

***Table 2.*** Effect of $GA_3$ and daminozide on the efficiency of somatic embryogenesis from immature embryos of spring wheat 'Andros' [a,b]

| Daminozide (mM) | $GA_3$ (μM) | Somatic embryogenesis (%) | | Shoots number | | Precocious germination (%) | |
|---|---|---|---|---|---|---|---|
| 0.0 | 0.00 | 75.3 | bcd | 12.3 | a | 10.0 | b |
| | 0.29 | 71.8 | bcde | 12.7 | a | 34.7 | ef |
| | 0.75 | 72.4 | bcde | 11.6 | ab | 51.6 | g |
| | 1.44 | 68.0 | cdef | 12.2 | a | 61.7 | hi |
| | 2.89 | 63.0 | def | 9.9 | bcd | 57.3 | gh |
| | 5.79 | 57.7 | ef | 10.2 | bc | 61.4 | hi |
| 0.255 | 0.00 | 91.7 | a | 10.2 | bc | 6.3 | ab |
| | 0.29 | 84.9 | ab | 9.4 | cde | 23.3 | cd |
| | 0.75 | 79.2 | abc | 7.8 | efg | 28.0 | de |
| | 1.44 | 72.5 | bcde | 8.3 | defg | 29.2 | de |
| | 2.89 | 62.9 | def | 8.7 | cdef | 35.0 | ef |
| | 5.79 | 58.3 | ef | 7.1 | fg | 62.5 | i |
| 0.510 | 0.00 | 90.3 | a | 8.0 | efg | 0.0 | a |
| | 0.29 | 79.5 | abc | 7.4 | fg | 19.6 | c |
| | 0.75 | 64.9 | cdef | 6.6 | g | 30.0 | de |
| | 1.44 | 63.8 | def | 6.9 | fg | 34.3 | ef |
| | 2.89 | 50.8 | f | 7.1 | fg | 32.0 | e |
| | 5.79 | 50.0 | f | 6.4 | g | 40.0 | f |

a - Explants were cultured on MS medium supplemented with 9.05 μM 2,4-D

b - Means within columns with the same letter are not significantly different at $\rho < 0.05$ according the Duncan's multiple range test (two-way Anova)

differentiation. Calli were incubated for 21 days under 16 hour illumination of 40 μmol $m^{-2}s^{1}$. In another experiment mature embryos were cultivated without daminozide on callus induction medium supplemented with 2,4-D (45.25 μM) and IAA (2.85 μM). 21 days later calli were transferred onto the auxine-free medium supplemented with daminozide (0, 0.064, 0.127, 0.255 or 0.510 mM) for 21 days cultivation under 16 hour illumination of 40 μmol $m^{-2}s^{1}$. In both experiments regeneration of plantlets was carried out in glass jars on hormone-free MS medium at 24-26$^0$C within 16 h/8 h light/dark cycle provided by white and fluora tubes (1500 lux).

***Statistical analysis***

At the end of callus induction phase the variable percentage (rate) of embryogenic callus formation (number of embryogenic calli / placed (initial) embryos × 100) was measured by counting per Petri dish. Each dish contained 25 explants (immature embryos) or 10 explants (mature embryos). Three dishes with three repetitions at least were analyzed. Embryogenic calli were grown in glass jars (10 embryogenic calli per jar) to promote regeneration. The average number of developed plantlets was assessed after 4 weeks of culture by counting well developed plantlets with the length over 2 cm per an embryogenic callus. Data were analysed by analysis of variances (ANOVA). Means were compared by Duncan's multiple range test at $P = 0.05$.

**Results**

***Effect of daminozide in combination with dicamba, p-CPA or 2,4-D on somatic embryogenesis from immature embryos***

The effect of daminozide in combination with dicamba, CPA or 2,4-D on somatic embryogenesis and plant regeneration is shown in Table 1. A clear difference in embryogenesis induction from immature embryos of 'Andros', was found between auxin-like substances. An equal embryogenesis rate of 73% or 74% was obtained on the medium supplemented with

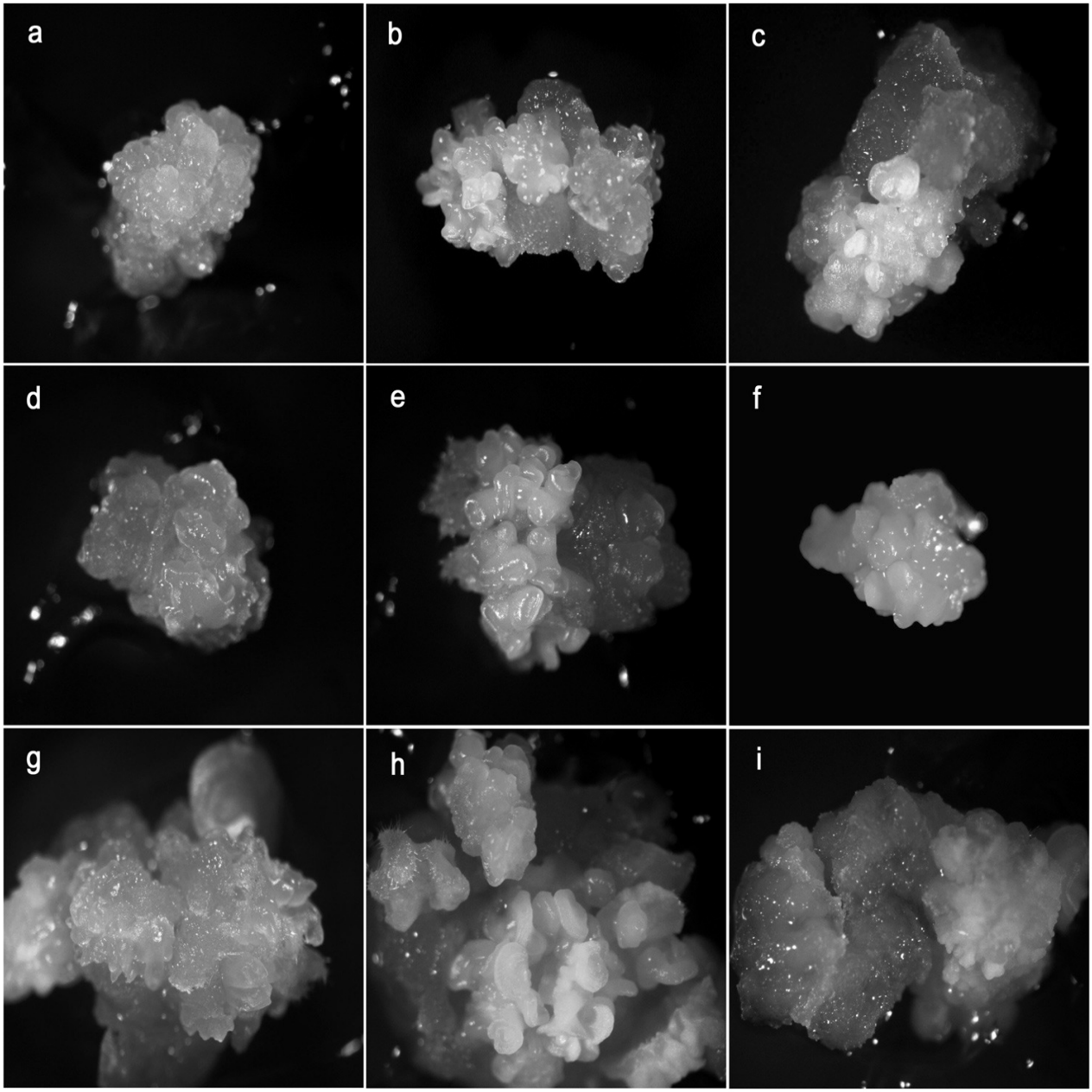


***Fig 1.*** The combined effect of daminozide and auxin-like substances on the somatic embryogenesis from immature embryos of wheat 'Andros', 23 days after culture initiation. Callus induction medium was supplemented with: (**a**) 9.05 µM 2,4-D, (**b**) 9.05 µM 2,4-D and 0.127 mM daminozide, (**c**) 9.05 µM 2,4-D and 1.019 mM daminozide, (**d**) 1.69 µM CPA, (**e**) 1.70 µM CPA and 0.127 mM daminozide, (**f**) 1.69 µM CPA and 1.019 mM daminozide, (**g**) 9.05 µM dicamba, (**h**) 9.05 µM dicamba and 0.127 mM daminozide, (**i**) 9.05 µM dicamba and 1.019 mM daminozide.

***Table 3.*** Effect of daminozide on efficiency of somatic embryogenesis and plant regeneration from zygotic embryos of spring and winter Russian wheat cultivars [a]

| Genotypes | Daminozide 0.000 mM | | Daminozide 0.127 mM | | Significance level, $p$ [b] | |
|---|---|---|---|---|---|---|
| | Somatic embryogenesis (%) | Shoot number | Somatic embryogenesis (%) | Shoot number | Somatic embryogenesis (%) | Shoot number |
| Spring genotypes | | | | | | |
| Andros | 74 | 12.9 | 92 | 11.1 | 0.0050 ** | 0.0951 ns |
| Enita | 95 | 15.3 | 100 | 16.7 | 0.0612 ns | 0.3903 ns |
| Noris | 92 | 13.7 | 98 | 13.8 | 0.0537 ns | 0.9655 ns |
| Lada | 89 | 16.0 | 97 | 18.5 | 0.0156 ** | 0.1423 ns |
| Priokskaya | 96 | 16.5 | 97 | 19.9 | 0.7178 ns | 0.0046 ** |
| Tajoznaja | 95 | 18.4 | 100 | 18.1 | 0.1665 ns | 0.9376 ns |
| Luba | 46 | 9.8 | 63 | 8.6 | 0.0221 ** | 0.6365 ns |
| Winter genotypes | | | | | | |
| Batko | 49 | 12.2 | 65 | 14.0 | 0.0251** | 0.1595 ns |
| Delta | 25 | 8.5 | 48 | 10.4 | 0.0038 ** | 0.2111 ns |
| Krasnodarskaya | 65 | 9.3 | 92 | 10.2 | 0.0458 ** | 0.4842 ns |
| Kroshka | 78 | 12.8 | 89 | 13.6 | 0.0873 ns | 0.2200 ns |

a - Explants were cultured on MS medium supplemented with 9.05 μM 2,4-D

b - Level of significance of the difference between two media for the studied genotype according the Duncan's multiple range test (** - indicate significant effect at $p < 0.05$, ns - non significant)

dicamba and 2,4-D, respectively, in the absence of daminozide. The rate of somatic embryogenesis on medium supplemented with *p*-CPA, was twice lower (37%). Explants cultured on medium supplemented with various concentration of daminozide started swelling after 5-7 days of culture. During the screening phase for callus induction two morphotypes of embryogenic calli usually were observed. Most of explants cultured on the media without daminozide formed off-white, compact, nodular embryogenic callus (Fig 1a, 1d, 1g). White, compact, nodular-organized structures along with off-white embryogenic callus were observed on the medium containing low daminozide concentrations (0.064 – 0.255 mM) (Fig 1b, 1e, 1h). Increase of daminozide concentration increased the portion of white embryogenic callus and altered callus properties. At the highest concentrations (0.765 and 1.019 mM) white callus became less organized and less compact.

A complete conversion of white embryogenic callus into white non-embryogenic clumps could be observed on some explants in the presence of 1.019 mM daminozide, especially on dicamba containing media (Fig 1e, 1f, 1i). The addition of low and moderate daminozide concentrations (0.064 – 0.510 mM) had a clear positive effect on the embryogenesis in the presence of all tested auxin-like substances. The best embryogenesis response was induced by daminozide at 0.127 – 0.255 mM. The combination of 2,4-D and CPA with daminozide at 0.127 mM increased the embryogenic ability of explants up to 91% and 55%, respectively. The most significant promotion of somatic embryogenesis on dicamba-containing medium, from 73% to 86%, was observed when the higher level of daminozide (0.255 mM) was applied.

The type of synthetic auxin used in combination with daminozide was found to influence the shoot production. In the absence of daminozide, the number of shoots resulted from embryogenic callus was higher for the explants cultured on dicamba and 2,4-D (12.9 and 11.1 shoots per explant). Explants cultured on CPA regenerated fewer plants (6.4 shoots per explant). It should to be noted that after transferring on regeneration medium near 10% of embryogenic callus cultured in the presence of dicamba regenerated from one to three albino plants among green shoots. The absence of albino regenerants from callus developed on 2,4-D or CPA medium pointed to the negative effect of dicamba on embryo morphology. Besides the promotive effect on the embryogenesis, the addition of daminozide had an inhibitory effect on the regeneration (Fig 2). The

average number of shoots developed per explants was lower than on the medium without daminozide, except three combinations of daminozide with *p*-CPA. On dicamba-containing medium, daminozide concentrations higher than 0.064 mM decreased the number of shoots per explant markedly. A significant reduction of shoot formation was observed when the highest concentration of daminozide (> 0.255 mM) was used coupled with both 2,4-D and CPA (Table 1). In the presented experiment the best embryogenesis response of 90-91% was observed at two daminozide concentration (0.127 or 0.255 mM) combined with 2,4-D. Although the percentage of embryogenic callus induction did not differ notably at those two levels, the average number of shoots produced per explant at lower daminozide concentration was found to be higher and did not significantly differ from the control (Table 1).

***Effect of daminozide in combination with $GA_3$ on somatic embryogenesis***

This experiment was carried out to improve the somatic embryo development and regeneration of plants from embryogenic callus under simultaneous application of daminozide and $GA_3$. Six $GA_3$ concentrations (0, 0.29, 0.75, 1.44, 2.89 or 5.79 μM) were combined with three daminozide concentrations (0, 0.255 or 0.510 mM). Generally the addition of $GA_3$ to callus induction medium inhibited the growth of callus regardless the presence or absence of daminozide. On basal medium without daminozide the increase of the $GA_3$ concentration decreased the percentage of embryogenic callus production from 75.3% (0 μM) to 57.7% (5.79 μM). The same observation was found for the average number of shoots that decreased from 12.3 to 9.9 shoots per explant. The number of explants showed the precocious germination was clearly increased along with the increase of $GA_3$ concentration. For example, only 10% of explants showed precocious germination on the basal medium without $GA_3$, while in the presence of 1.44-5.79 μM $GA_3$ more than half of cultured immature embryos germinated and developed main embryo shoot. The addition of daminozide to the medium supplemented with $GA_3$ decreased the rate of precocious germination, although did not prevent it completely (Table 2).

No positive effects on the somatic embryogenesis were found when daminozide and $GA_3$ were combined. The combination of moderate and high concentrations of $GA_3$ with daminozide resulted in browning and partial necrosis of embryogenic clumps by the end of cultivation. The higher concentration of $GA_3$ and daminoside was used for combination, the stronger inhibition of the embryogensis was observed. Any combination of $GA_3$ and daminozide was followed by the decrease in a number of shoots. Due to the absence of the positive influence of $GA_3$ on the wheat somatic embryogenesis in the next experiments daminozide was used alone.

***Influence of daminozide on somatic embryogenesis of Russian winter and spring wheat genotypes***

Significant differences were observed within immature embryos of eleven Russian genotypes tested for embryogenic capacity on two media (Table 3). The first medium used as a control was supplemented with 9.05 μM 2,4-D. The second medium contained the combination of 0.127 mM daminozide and 9.05 μM 2,4-D. Large number of variation observed on the control medium. Among seven spring type genotypes, 'Priokskaya', followed by 'Tajoznaja' and 'Enita', showed the highest percentage of embryogenic callus production in the range of 95-96%. The best results were obtained for winter type genotypes 'Kroshka' and 'Krasnodarskaya' (78% and 65% of embryo- genesis rate, respectively). Most of varieties produced from 12 to 18 shoots per embryogenic calli. Less than 10 shoots were regenerated from calli of 'Krasnodarskaya' and 'Delta' (winter wheat) or 'Luba' (spring wheat).

The combination of 0.127 mM daminozide and 9.05 μM 2.4-D stimulated higher embryogenic response of immature embryos for all tested wheat genotypes (Table 3). The effect of daminozide was more evident for genotypes with lower embryogenic capacity. On daminozide containing medium, the spring varieties 'Tajoznaja' and 'Enita' showed the highest percentage of embryogenic callus production. Values close to 100% were also observed for 'Norris', 'Lada' and 'Priokskaya' (98%, 97% and 97% of embryo-genesis rate, respectively). The percentage of embryogenic callus production for winter varieties 'Krasnodarskaya' and 'Kroshka' increased from 65% and 78% to 92% and 89%, respectively. No statistically significant differences in number of shoots per embryogenic callus were found for ten genotypes between two media studied. Daminozide only proved to improve significantly regeneration of

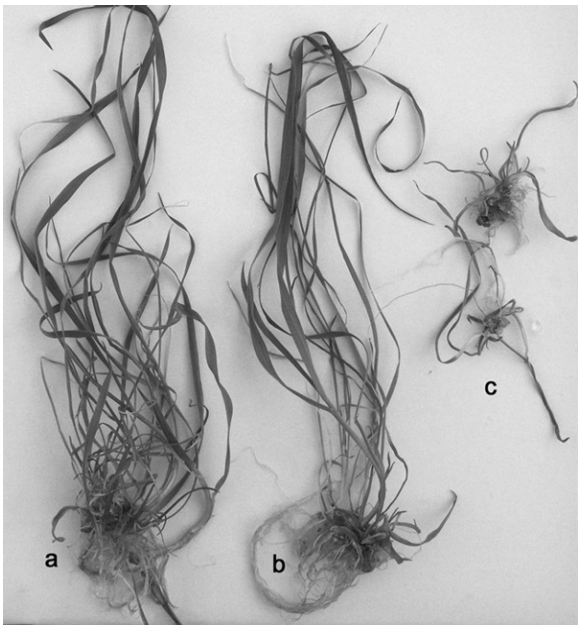


***Fig 2.*** The effect of daminozide on the shoot formation from callus produced from immature embryos cultured on MS medium supplemented with (a) 9.05 μM 2,4-D, (b) 9.05 μM 2,4-D and 0.255 mM daminozide, (c) 9.05 μM 2,4-D and 1.019 mM daminozide.

spring variety 'Priokskaya' (from 16.5 to 19.9 shoots per callus). In contrast, most of genotypes produced near the same number of shoots per embryogenic callus compared to control medium (Table 3).

***Effect of daminozide on somatic embryogenesis from mature embryos of wheat***

An experiment was carried out with the spring variety Tajoznaja that responded well to culture conditions in previous experiments (Filippov et al., 2006). Since the protocol for somatic embryogenesis from mature embryos used in our study included consecutive culturing of explants on callus induction medium followed by differentiation medium, daminozide was added to one of the media once. The effect of different concentrations of daminozide on the somatic embryogenesis of mature embryos is shown in table 4. Callus formation from mature embryos started after two or three days after culture initiation. On the 7-10-th day the callus induction rate reached 100%. By the end of callus induction stage the average diameter of calli from mature seeds attained to 5-10 mm. Callus was friable, yellowish-white. Some organi- zation appeared as smooth portions with visible slightly nodular embryogenic structures. After transfering to auxin-free medium differentiation via somatic embryogenesis was observed within 5-15 days later. By that time transferred callus doubled in size and consisted mostly of watery white-yellow soft structures. Well developed single somatic embryos, small groups of somatic embryoids and young plantlets can be found by the end of cultivation on differentiation medium. Usually, first evidence of plant development was visible after a week cultivation of embryogenic callus on differentiation medium under the light. Unlike the immature zygotic embryos, the mature embryos showed much lower embryogenesis. Only 54.4% of cultured mature embryos formed embryogenic callus on daminozide-free medium, while the immature embryos of 'Tajoznaja' showed a higher embryogenesis about 95% (Table 3). As compared with the explants cultured on daminozide-free medium, the presence of daminozide in callus induction medium did not influenced significantly on the rate of embryogenesis and the average number of shoots produced per explant (Table 4). On the other hand, the presence of daminozide in the different- tiation medium had negative effect on the production of embryogenic callus (Table 4). The number of explants that produced embryogenic callus was reduced from 54.4 % at the control to a range of 46.4-33.0% in the presence of daminozide. In spite of some negative effect of daminozide on embryo- genesis, there was no significant difference in the number of shoots produced per explant cultured on all media. The number of shoots generated by embryogenic callus varied from 5.7 to 7.2 plantlets per explants.

**Discussion**

In the present study it was found that the GAs–biosynthesis inhibitor, daminozide, influenced the efficiency of somatic embryogenesis and plant regeneration from immature zygotic embryos of wheat. Low and medial daminozide concentrations were able to increase the percentage of embryogenic callus formation. The stability of daminozide effects on media contained different types of synthetic auxins indicated the uniform mode of action in the presence of those substances. However the type of auxin-like

substances used for combination with daminozide had a certain effect on morphology of callus and its development. In our study an equal embryogenic response was observed on the medium containing 2,4-D and dicamba. Similar results were also reported by Redway et al., (1990). At the contrary Hunsinger and Schauz (1987) and Carman et al., (1987) reported that dicamba significantly increased the number of embryoids when compared to 2,4-D. In our experiments such effect was not found, however, the tendency to form embryos and plants more rapidly on dicamba was observed. Up to now *p*-CPA has only been used in a few experiments on the induction of somatic embryogenesis. For wheat this substance was used by Lazar et al., (1983) to induce callus on mature embryos. In our study *p*-CPA was much less effective than 2,4-D and dicamba for stimulation the embryogenesis response from immature embryos of 'Andros'. Daminozide is well known as plant growth retardant that controls the vegetative and reproductive growth and is frequently commercially used in ornamental plants to modify the stem length and shape of plants (Kuehny et al., 2001; Lewis et al., 2004). From this point it is reasonable to observe the delay of development of wheat somatic embryos into shoots in the presence of daminozide. According to some reports it acts on the late stages of gibberellin metabolism and blocks particularly 3 beta-hydroxy-lation, thereby inhibiting transformation from inactive precursors into active GAs forms (Brown et al., 1997; Rademacher 2000). However, other data indicates that daminozide may not inhibit the responses to $GA_9$ or $GA_{20}$ by blocking gibberellin hydroxylation (Menhenett, 1982). Despite the specific modes of action of daminozide still remains to be unclear; it could restrict, wholly or partly, the metabolism or action of one or more active endogenous gibberellins.

Our attempts to stimulate the development of somatic embryos by application of exogenous $GA_3$ did not bring to success. When $GA_3$ was used in combination with daminozide, it did not promote the development of embryos. We also found that the higher exogenous $GA_3$ level was present in the initiation medium the more frequent precocious germination of explants was observed. This resulted in lower morphogenic response. Addition of daminozide to $GA_3$ containing medium partially decreased the precocious germination, but did not prevented it completely. Those observation indicated that daminozide and $GA_3$ could not be recommended as compensative substances for wheat and that their interactions may include different metabolic pathways besides the gibberellins metabolism.

Previously both negative and positive reaction upon treatment with $GA_3$ and gibberellins biosynthesis inhibitors was reported in *in vitro* tissue culture. GA-inhibitors positively affected on the embryogenic capacity of carrot (Fujimura and Komamine 1975, Tokuji and Kuriyama 2003), *Geranium* (Hutchinson et al., 1997; Senaratna et al. 2001), *Asparagus* (Feng and Wolyn 1993), *Oncidium* (Chen and Chang 2003) and *Arabidopsis* (Ezura and Harberd 1995), whereas the exogenous GAs application was found to retard embryogenesis in those species. Application of daminozide among the others gibberellins bio-synthesis inhibitors caused statistically significant increases in initiation of somatic embryogenesis of loblolly pine (Pullman et al. 2005). Also daminozide was successfully used to initiate embryogenic cultures from undeveloped ovules of *Citrus* (Gmitter and Moore 1986). Quite the contrary, the addition of $GA_3$ to induction medium stimulated morphogenic response in petiole-derived tissue cultures of *Medicago sativa L.* (Rudus et al. 2002) and fennel (Hunault and Maatar 1995), whereas exogenous $GA_3$-ihibitors suppressed the callus growth of *Medicago sativa L.* during the differentiation stage (Rudus et al., 2002).

Our results indicated that daminozide at low concentrations can increase embryogenic response of wheat cultivars, especially in cultivars with initially low embryogenesis. According to numerous reports model wheat varieties, for example 'Bobwhite' or 'Chinese Spring', have a relatively high embryogenic capacity. Elite locally grown varieties adapted to central Europe and North America, however, have a much lower response in tissue culture (Maddock et al., 1983; He at al., 1988; Fennell et al., 1996; Viertel et al., 1998). Here we have obtained similar data with spring and winter cultivars grown in Russia, however our results indicate that genotype variations can be partially overcome by the daminozide application. The addition of daminozide to 2,4-D containing medium generally increased the embryogenic competence of immature embryos of eleven varieties. The addition of daminozide stimulated the formation of white compact, nodular-organized callus on most of studied varieties, whereas mostly off-white embryo- genic structures were formed on callus induction medium without daminozide.

The positive effects of daminozide on wheat genotypes indicate that besides the inhibitory activity

***Table 4.*** Effect of daminozide on the efficiency of somatic embryogenesis from mature embryos of wheat 'Tajoznaja'.

| Concentration of daminozide (mM) | Somatic embryogenesis (%) | | Shoot number |
|---|---|---|---|
| 0.00 (control ) [a] | 54.4 | a | 7.2 a |
| Callus induction medium [b] | | | |
| 0.127 | 47.8 | ab | 6.1 a |
| 0.255 | 52.8 | a | 6.5 a |
| 0.510 | 49.4 | a | 6.8 a |
| 1.019 | 51.7 | a | 7.2 a |
| Differentiation medium [c] | | | |
| 0.064 | 41.1 | ab | 6.1 a |
| 0.127 | 46.4 | ab | 7.1 a |
| 0.255 | 42.8 | ab | 6.9 a |
| 0.510 | 33.0 | b | 5.7 a |

[a] – Mature embryos cultured for 20 days on callus induction medium (MS supplemented with 45.25 μM 2,4-D and 2.85 μM IAA), then were transferred to differentiation medium (hormone-free MS medium).
[b] – Daminozide was added to the callus induction medium, differentiation medium was not supplemented with daminozide
[c] – Mature embryos cultured for 20 days on callus induction medium without daminozide, and then calluses were transferred to the differentiation medium supplemented with daminozide.
* - Means within columns with the same letter are not significantly different at $\rho < 0.05$ according the Duncan's multiple range test (one-way Anova)

in GAs–biosynthesis, daminozide or its plant metabolites may also influence the distribution and synthesis of other plant growth regulators controlling *in vitro* developmental processes. Earlier a considerable increase of peroxidase and IAA-oxidase activities in the tissues of tobacco plants treated with daminozide was found by Petkova and Angelova (1995). Daminozide inhibited ethylene production in apple fruit by blocking the conversion of methionine to aminocyclopropane-1-carboxylic acid (Gussman at al. 1993) and affected fruit-set maturity, fruit coloring, total chlorophyll and carotenoids content of tomato (Gabr et al. 2006). All those observations suggest the complexity of direct and non-direct action of daminozide in tissue culture, especially on the endogenous level of different natural phytohormones. Unlike the immature embryos, the mature ones reacted neutral or negative on the presence of daminozide in medium. This difference might be associated with the different initial balance of endogenous growth regulators in mature and immature tissues that is the consequence with the differences in physiological states. For this reason mature embryos require the application of other growth regulators combinations for improvement of somatic embryogenesis.

As far as we know, this is the first report of the effect of GAs–biosynthesis inhibitors on the efficiency of somatic embryogenesis for cereals. In the present work modification of callus induction medium by addition of certain daminozide concentration seemed to be very effective for induction of high embryogenic activity of wheat immature embryos. To clarify whether daminozide can affect the initial physiological state of explants or induce the genetic programs responsible for embryogenesis, a more detailed study is required. However the practical conclusion from the results presented here, is that daminozide can increase the embryogenesis efficiency of varieties with low embryogenic potential. This might permit the application of this modified protocol to a broader spectrum of cereals genotypes for different biotechnology applications.

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