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Increasing the frost resistance of 'Golden Delicious', 'Gala' and 'Šampion' apple cultivars

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ABSTRACT

The aim of this research was the evaluation of whether the application of 5-ALA, which has potential as a plant growth stimulating agent with indirect anti-stress activity, can limit the damage caused by low temperatures in winter. The research was conducted on frost-sensitive apple cultivars for three years in an orchard and in a laboratory. During the vegetative seasons of each year, the trees of three apple cultivars ('Golden Delicious', 'Gala' and 'Šampion') were subject to 5-ALA foliar application. Three times during each winter following the field application, apple shoots were taken to the laboratory and frozen at -20ºC, -25ºC and -30ºC. After incubation, the damage to the sensitive parts of the shoots was evaluated. The application had a positive effect on the frost resistance of all cultivars, most often at the end of dormancy, which is especially noteworthy because frost damage occurs most frequently during this period. Positive effects of the application were found in most of the experiment combinations of cultivar, year, freezing date and shoot part. Additionally, the positive influence of 5-ALA application on frost resistance was found to increase with each year of the research.

Key words: 5-ALA, endogenous application, frost risk , survival test

INTRODUCTION

Photosynthesis is the basis of the growth and development of autotrophs. In photosynthetic organisms, chlorophyll biosynthesis is tightly regulated at various levels in response to environmental conditions and plant development. The synthesis of chlorophyll is a complicated multistage biochemical process taking place in a cell. One of the last stages of chlorophyll synthesis is the joining of molecules of the 5-aminolevulinic acid (5-ALA) in a porphyrin ring. 5-Aminolevulinic acid, naturally occurring in plant compounds (Morales-Payan and Stall 2005), is a keto amino acid with a molecular weight of 131. It is a precursor of heme, chlorophyll, vitamin B12, and other in vivo tetrapyrrole compounds (Stobart and Ameen-Bukhari 1984, Watanabe et al. 2006).

5-ALA is synthesized from glutamate in a reaction involving a glutamyl tRNA intermediate and requiring ATP and NADPH as co-factors or through another pathway from succinyl CoA and glycine (Beale 1990, Sasaki et al. 2002). Considering its role as a precursor of chlorophyll, 5-ALA is considered to be the only amino acid directly related to photosynthesis in which it is

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stage-limiting in the biosynthesis of chlorophyll (von Wettstein et al. 1995, Hotta et al. 1997, Memon et al. 2009). The natural concentrations of 5-ALA found in plants are regulated *in vivo* at a low level of ca. 60 µmol (Zhang et al. 2006).

Since the mid-1990s, research aimed at determining the influence of applying exogenous 5-aminolevulinic acid to humans and to plants has been conducted in many countries worldwide. 5-ALA has been applied in medicine, in the so-called photodynamic diagnosis (PDD) and photodynamic therapy (PDT) of cancer (Peng et al. 1997, Fukuda et al. 2005). Considering its stimulating effect on the accumulation of an excess amount of protoporphyrin IX, which produces a large amount of reactive oxygen species through a photodynamic mechanism, it has also been reported to act as a natural photodynamic herbicide when applied to a plant at a high concentration of more than 10 mM (Hotta et al. 1997, Choi et al. 1999).

However, experiments carried out in the last decade show that exogenous 5-ALA, used in low concentrations, has potential as a plant growth stimulating agent with indirect anti-stress activity (Wang et al. 2005, Łysiak and Kurlus 2008, Pavlovic et al. 2009, Averina et al. 2010, Hara et al. 2010, Naeem et al. 2010, Smoleń et al. 2010). This growth promotion is probably related to the photosynthesis-enhancing effects of 5-ALA, which have been demonstrated in various plant species. However, further mechanisms of the growth-promoting effects of 5-ALA have not been elucidated (Hara et al. 2010).

Applying exogenous 5-aminolevulinic acid to plants as a fertilizer by way of either foliar or soil treatment has been widely tested. Soil treatment allows the limitation of the influence of some stress conditions. Watanabe et al. (2000) proved that the foliar treatment of 5-aminolevulinic acid increased the salt tolerance of cotton seedlings in Japan. In Saudi Arabia, Watanabe et al. (2004) used drip irrigation to improve the salt tolerance of tomato and wheat seedlings. The level of 5-aminolevulinic acid influences many cell processes. It can directly affect the resistance of plants to damage, as shown by Hodgins and Hustee (1986), who conducted research on frost damage to maize, and by Łysiak and Kurlus (2009) in pear trees. The increase in frost resistance in winter is a multistage process which is directly connected to plant health, vigour and condition. One of the important stages consists of deep changes in the cell structure that require the synthesis of nucleic acids, and later of enzymes, which is very energy consuming. This process can take place undisturbed only in well-nourished cells that contain large amounts of nutrient reserves (Hołubowicz 1984).

Frost damage to fruit trees is one of the basic factors limiting the production of many fruit species in Poland. Both winter and spring frost damage can reduce crops as much as by 50%. Therefore, research aiming at discovering how fruit cultivars become frost resistant allows us to find methods of increasing frost resistance in the basic cultivars grown in Poland and is thus of great economic value. These considerations provided motivation for the Faculty of Horticulture in Poznan to conduct an experiment whose goal was to assess the influence of foliar application of 5-aminolevulinic acid on the frost resistance of one-year-old shoots, buds and bud bases of apple trees.

MATERIAL AND METHODS

The experiment was set up in western Poland at the Research Station of the Poznan University of Life Sciences (52°31' north latitude and 16°38' east longitude) on a grey-brown podzolic soil overlaying light boulder clay. Three cultivars were used in the experiment: 'Golden Delicious', 'Gala' and 'Šampion', grown on M.9 rootstock in rows, with 4 m spacing between rows, and 1.25 m ('Šampion') and 1.5 m ('Golden Delicious', 'Gala') spacing between trees in a row. Trees were at full cropping capacity at the time of the experiment ('Šampion' was planted in 1997 and other cultivars in 1998). The trees were of the same height and in good condition. The orchard was protected and maintained (pest, disease, fertilization, irrigation and weed control) in line with the recommendations for commercial orchards.

The experiment was conducted in the years 2006-2009 and was composed of three one-year cycles, each of which was divided into a field and laboratory part.

Field part

During each of the three growing seasons (2006, 2007 and 2008), the trees were treated nine times with foliar sprays of 5-ALA in a concentration of 100 ppm applied along with nitrogen (9.5%), magnesium (5.7%), manganese (0.3%) and boron (0.45%). The treatments started about one week before the full bloom period and were repeated seven times in one-week intervals (the last treatment was carried out in the second half of June, at the end of

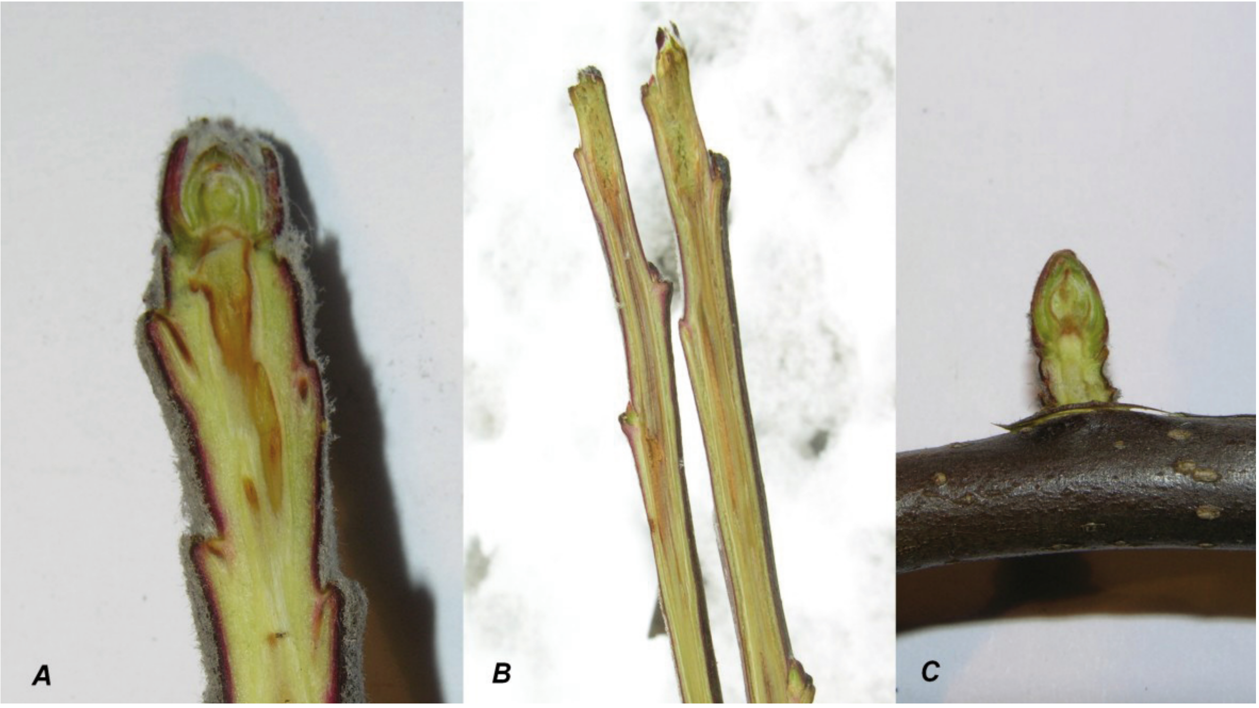

Figure 1. Winter damage in: A) bud base and wood, B) shoots, C) flower bud

the strong growth of shoots). An additional autumn spray with the same concentration was performed at the end of September. The water amount used for the applications equalled 1000 l per hectare.

The control group of trees, which comprised 64 trees of each cultivar, was subjected to the same agricultural practices except for 5-ALA application.

Laboratory part

Shoots were taken three times (in December, in February and in March) each year from all trees (evenly from all sides of the canopy and different heights). Next, the shoots were tested for survival by freezing them at temperatures of -20°C, -25°C and -30°C. After freezing, the shoots were placed in peat substrate in a greenhouse at a temperature of 21°C and in high humidity. After two weeks of incubation, frost damage to three indicating parts – buds, wood tissue and bud base sectors – of one-year-old shoots was examined (Fig. 1).

Additionally, shoots from the orchard which had not been subject to freezing were assessed. The temperatures in the orchard in the winter seasons 2006/2007, 2007/2008 and 2008/2009 are presented in Figure 2. Frost damage was measured according to the 5-point damage scale proposed by Hołubowicz (1984) where 1 means no damage symptoms in the form of tissue discolouration and 5 means irreparable damage leading to the death of plant parts. Frost damage was measured on buds and bud bases frozen on all three dates and for each cultivar. Three persons experienced in evaluating frost damage assessed the condition of all plant parts by comparing them against the model developed by Hołubowicz (1984). The average of the three ratings was treated as one measurement and was subject to statistical analysis. Each measured sample consisted of 60 shoot parts (buds or bud bases, respectively). Frost damage to shoots was measured on a sample of 20 shoots in each combination.

Data obtained from the biometric measurements were submitted to the three-way analysis of variance (ANOVA) in order to examine differences in means among the species studied. If critical differences were noted, multiple comparisons were carried out based on Duncan's test (p = 0.05).

RESULTS

All of the basic factors – namely, the cultivar, the examined plant part, the freezing date and the temperature – affected the effectiveness of the treatment (Tabs 1-3).

The assessment of the damage to the flower buds – both in laboratory and field conditions – showed a favourable influence of 5-ALA treatment on frost resistance (Tab. 1). The general mean results for all years, temperatures and cultivars showed a significant difference between the treated and untreated samples. In field conditions, the most severe damage was found in the buds of 'Golden

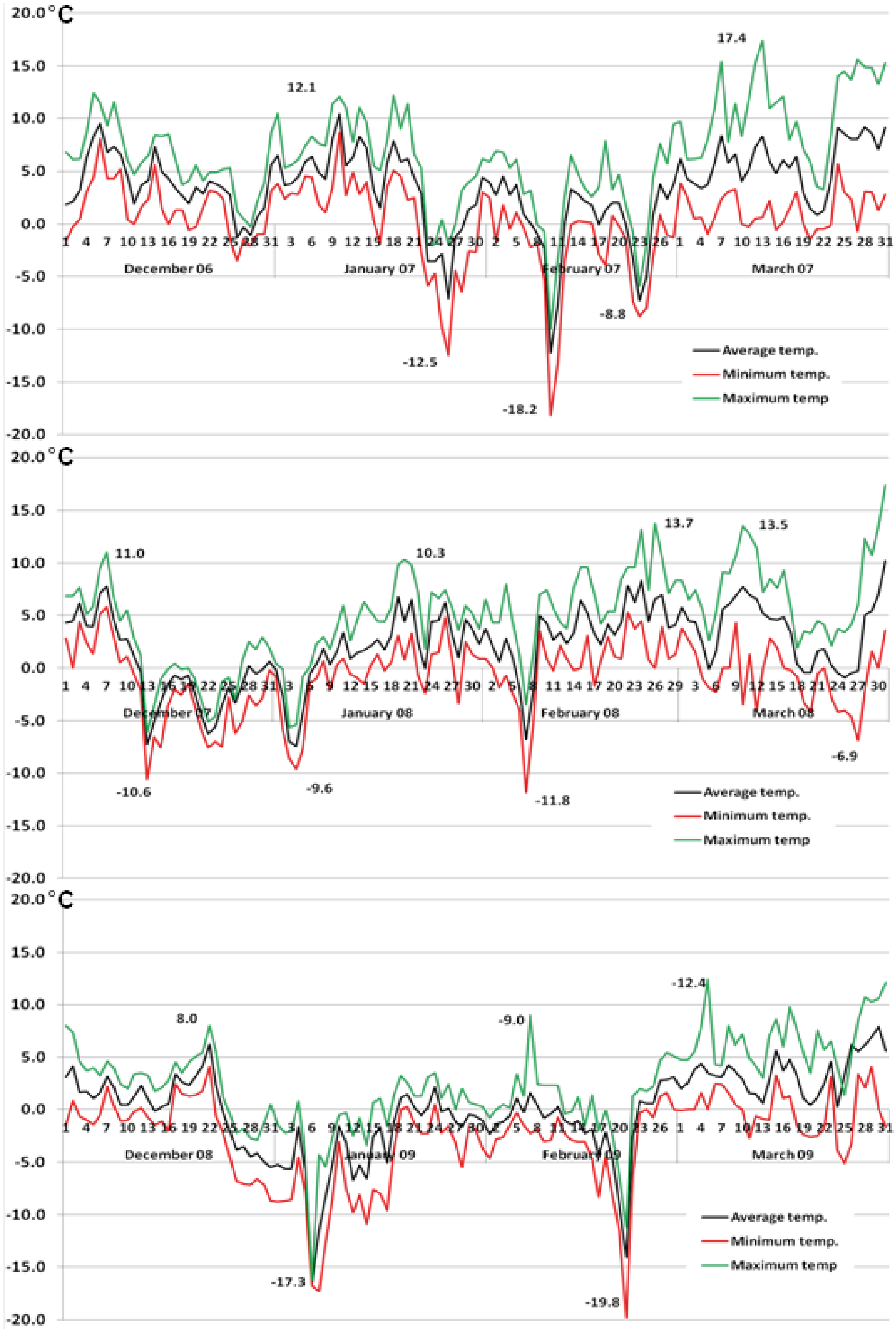

Figure 2. Air temperature in the orchard during the winter months in 2006-2009 measured at 2 m height

Delicious', although the buds of 'Šampion' were damaged most frequently, i.e. on all dates of the experiment except December 2006 and 2008. In the months in which frost damage occurred in field conditions (mostly in February), frost damaged all cultivars on 15 measurement dates. On eight of those dates, 5-ALA sprayed buds were significantly less damaged than the unsprayed ones, and on the rest of those dates the differences were insignificant.

The assessment of the buds frozen at low temperatures in laboratory conditions showed that the freezing temperature was the most important factor and the date of the experiment the second-most important factor affecting frost damage (Tab. 1). A negative effect of 5-ALA application was observed only for 'Gala' buds during the first freezing at -20°C in December of the first year of the experiment. Lower temperatures applied on the same date killed all buds and there were no differences as regards frost resistance between buds treated and not treated with 5-ALA. Despite the aforementioned (only) negative response of

Table 1. Degree of damage to the flower buds of three apple cultivars

	Golden Delicious									
	Field conditions		Freezing temperature (°C)						Mean	
			-20		-25		-30			
	Ctr*	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA
Dec 06**	1.00 a***	1.00 a	1.33 bc	1.65 ef	4.93 xy	5.00 y	5.00 y	4.78 wx	3.07 gh	3.11 hi
Feb 07	2.03 gh	1.15 ab	2.63 j	2.78 jkl	4.20 st	3.32 nop	4.63 vw	3.95 r	3.37 j	2.80 de
Mar 07	2.09 h	1.05 a	3.14 mn	2.37 i	5.00 y	4.26 t	5.00 y	5.00 xy	3.81 l	3.16 hi
Dec 07	1.00 a	1.00 a	2.41 i	1.96 gh	2.93 klm	2.98 lm	3.44 opq	2.95 lm	2.44 c	2.22 b
Feb 08	1.00 a	1.00 a	1.43 cd	1.00 a	5.00 y	2.95 lm	5.00 y	3.45 pq	3.11 hi	2.10 a
Mar 08	1.00 a	1.00 a	3.24 no	1.58 de	4.63 vw	4.35 tu	5.00 y	5.00 y	3.47 k	2.98 fg
Dec 08	1.00 a	1.00 a	2.73 jk	2.37 i	4.03 rs	3.86 r	4.50 uv	4.36 tu	3.06 gh	2.90 ef
Feb 09	1.00 a	1.00 a	1.93 gh	1.00 a	3.53 q	2.30 i	5.00 y	5.00 y	2.86 e	2.31 b
Mar 09	1.00 a	1.00 a	1.83 fg	1.00 a	5.00 y	3.86 r	5.00 y	5.00 y	3.21 i	2.71 d
Dec mean	1.00 a	1.00 a	2.15 b	1.99 b	3.96 c	3.95 c	4.31 d	4.03 c	2.86 a	2.74 a
Feb mean	1.34 b	1.05 a	1.99 d	1.59 c	4.24 f	4.86 e	4.88 g	4.11 f	3.11 b	2.40 a
Mar mean	1.36 b	1.01 a	2.73 d	1.65 c	4.86 f	4.15 e	5.00 f	5.00 f	3.49 b	2.95 a
General mean	1.24 b	1.02 a	2.29 d	1.74 c	4.36 f	3.65 e	4.73 g	4.37 f	3.15 b	2.70 a
	Gala									
Dec 06	1.23 ab	1.05 a	1.93 def	4.58 pqr	4.98 u	5.00 u	5.00 u	5.00 tu	3.28 i	3.90 k
Feb 07	1.73 d	1.15 ab	1.93 def	1.48 c	3.33 lm	1.93 def	4.43 opq	4.25 o	2.85 f	2.20 b
Mar 07	1.23 ab	1.00 a	1.93 def	1.18 ab	3.42 m	2.78 i	4.83 stu	3.15 jkl	2.85 f	2.02 a
Dec 07	1.00 a	1.00 a	2.00 efg	1.20 ab	5.00 u	4.96 tu	5.00 u	5.00 tu	3.26 i	3.03 gh
Feb 08	1.00 a	1.00 a	1.00 a	1.00 a	4.80 rstu	2.37 h	5.00 u	5.00 tu	2.96 g	2.33 c
Mar 08	1.00 a	1.00 a	3.23 klm	1.98 efg	4.80 rstu	1.30 bc	5.00 u	4.96 tu	3.51 j	2.31 bc
Dec 08	1.00 a	1.00 a	2.13 fg	1.78 de	4.73 rst	2.98 ij	4.63 qrs	4.35 op	3.13 h	2.53 d
Feb 09	1.23 ab	1.38 bc	1.00 a	1.00 a	3.93 n	2.18 gh	4.73 rst	4.45 opq	2.72 e	2.25 bc
Mar 09	1.73 d	1.00 a	1.73 d	1.48 c	4.73 rst	3.08 jk	5.00 u	5.00 tu	3.30 i	2.63 de
Dec mean	1.09 a	1.02 a	2.02 b	2.52 c	4.90 e	4.31 d	4.88 e	4.75 e	3.22 a	3.15 a
Feb mean	1.33 a	1.18 a	1.32 a	1.16 a	4.02 c	2.18 b	4.72 d	4.55 d	2.84 b	2.67 a
Mar mean	1.33 b	1.00 a	2.29 c	1.54 b	4.32 d	2.38 c	4.94 e	4.35 d	3.22 b	2.32 a
General mean	1.25 b	1.06 a	1.87 d	1.74 c	4.41 f	2.95 e	4.84 h	4.55 g	3.09 b	2.58 a
	Šampion									
Dec 06	1.00 a	1.00 a	2.73 m	1.58 efgh	5.00 x	5.00 x	5.00 x	4.86 wx	3.44 hi	3.11 ef
Feb 07	1.45 cdef	1.63 efgh	2.93 mno	2.80 mn	4.00 st	3.90 rs	4.63 vw	3.95 rs	3.25 fg	3.07 e
Mar 07	1.80 ghij	1.00 a	2.93 mno	1.78 ghij	3.13 opq	2.68 m	5.00 x	5.00 x	3.21 fg	2.60 c
Dec 07	1.13 ab	1.00 a	2.03 jkl	1.38 bcde	1.43 cdef	1.68 fghi	5.00 x	5.00 x	2.39 b	2.25 a
Feb 08	1.80 ghij	1.00 a	1.03 a	1.00 a	5.00 x	3.35 q	5.00 x	5.00 x	3.21 fg	2.58 c
Mar 08	1.53 efg	1.00 a	3.03 nop	2.73 m	4.24 tu	3.95 rs	5.00 x	4.76 wx	3.45 hi	3.11 ef
Dec 08	1.00 a	1.00 a	3.70 r	2.10 kl	4.43 uv	3.70 r	5.00 x	5.00 x	3.54 i	2.94 d
Feb 09	2.15 l	1.83 hijk	2.10 kl	1.20 abcd	4.03 st	1.88 ijkl	5.00 x	5.00 x	3.32 gh	2.46 bc
Mar 09	1.83 hijk	1.55 efgh	2.03 jkl	1.48 def	3.23 pq	1.98 jkl	5.00 x	5.00 x	3.02 de	2.49 bc
Dec mean	1.06 a	1.00 a	2.82 c	1.68 b	3.62 d	3.46 d	5.00 e	4.94 e	3.13 a	2.77 a
Feb mean	1.80 b	1.49 a	2.02 b	1.67 a	4.34 d	3.04 c	4.88 e	4.64 e	3.25 b	2.69 a
Mar mean	1.72 b	1.18 a	2.66 d	1.99 c	3.51 e	2.87 d	5.00 f	4.91 f	3.22 b	2.73 a
General mean	1.49 b	1.24 a	2.50 d	1.78 c	3.83 f	3.12 e	4.96 h	4.81 g	3.19 b	2.74 a

*Ctr – Control, ALA – 5-aminolevulinic acid treatment
**Abbreviations means month and year of tree part examination
***Values followed by the same letter do not significantly differ at $p < 0.05$ after three-way ANOVA, except month means and general means (last four rows and last two columns), which were subjected to two-way ANOVA

Table 2. Degree of damage to the flower bud bases of three apple cultivars

	Golden Delicious									
	Field conditions		Freezing temperature (°C)						Mean	
			-20		-25		-30			
	Ctr	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA
Dec 06	1.00 a	1.00 a	1.98 d	1.95 d	4.65 p	4.13 mn	4.70 p	4.55 op	3.09 ij	2.09 fg
Feb 07	1.23 abc	1.15 ab	2.42 ef	2.29 e	5.00 q	3.40 i	5.00 q	5.00 q	3.41 k	3.95 gh
Mar 07	1.50 c	1.30 bc	2.70 g	2.38 ef	5.00 q	3.75 jk	5.00 q	5.00 q	3.55 l	3.10 ij
Dec 07	1.00 a	1.00 a	1.05 ab	1.20 ab	4.20 mn	4.35 no	4.50 op	4.15 mn	2.69 cde	2.67 cd
Feb 08	1.00 a	1.00 a	1.00 a	1.00 a	4.15 mn	4.30 mno	5.00 q	5.00 q	2.79 def	2.81 efg
Mar 08	1.00 a	1.00 a	3.03 h	1.98 d	3.83 kl	3.35 i	5.00 q	5.00 q	3.22 j	2.82 efg
Dec 08	1.00 a	1.00 a	2.33 e	1.25 abc	3.53 ij	3.45 i	4.70 p	4.65 p	2.89 fg	2.59 bc
Feb 09	1.00 a	1.00 a	2.63fg	1.00 a	4.03 lm	2.55 efg	4.55 op	2.98 h	5.05 hi	1.88 a
Mar 09	1.00 a	1.00 a	2.83 gh	1.00 a	3.83 kl	2.98 h	5.00 q	5.00 q	3.17 ij	2.48 b
Dec mean	1.00 a	1.00 a	1.80 c	1.47 b	4.13 d	3.96 d	4.63 e	4.45 e	2.89 a	2.72 a
Feb mean	1.08 a	1.05 a	2.02 c	1.43 b	4.39 e	3.42 d	4.84 f	2.49 e	3.08 b	2.55 a
Mar mean	1.18 a	1.10 a	2.85 c	1.78 b	4.22 e	4.36 d	5.00 f	5.00 f	3.31 b	2.81 a
General mean	1.10 a	1.05 a	2.22 c	1.56 b	4.24 e	3.58 d	4.83 g	4.67 f	3.09 b	2.69 a
	Gala									
Dec 06	1.00 a	1.00 a	1.63 b	3.16 defg	4.90 m	4.78 m	4.90 m	5.00 m	3.11 f	3.47 g
Feb 07	1.00 a	1.00 a	1.73 b	1.68 b	3.33 fg	1.96 c	4.90 m	4.86 m	2.74 d	2.37 a
Mar 07	1.00 a	1.00 a	1.00 a	1.00 a	4.34 l	3.38 g	5.00 m	4.86 a	2.85 d	2.56 bc
Dec 07	1.18 a	1.00 a	3.93 ij	2.09 c	5.00 m	4.18 kl	5.00 m	5.00 m	3.77 h	3.05 ef
Feb 08	1.00 a	1.00 a	1.03 a	1.00 a	4.82 m	3.13 def	5.00 m	5.00 m	2.97 e	2.52 bc
Mar 08	1.00 a	1.00 a	3.60 h	3.23 efg	4.24 kl	3.18 dfg	5.00 m	5.00 m	3.47 g	3.09 f
Dec 08	1.18 a	1.00 a	3.74 hi	2.10 c	4.04 jk	3.08 de	4.93 m	3.65 h	3.47 g	2.46 ab
Feb 09	1.00 a	1.00 a	1.00 a	1.00 a	4.83 m	3.38 g	5.00 m	5.00 m	2.97 e	2.58 c
Mar 09	1.98 c	1.73 b	2.23 efg	2.18 c	4.03 jk	2.98 d	5.00 m	5.00 m	3.55 g	2.96 e
Dec mean	1.13 a	1.00 a	3.09 c	2.45 b	4.65 e	4.01 d	4.94 f	4.52 e	3.45 b	2.99 a
Feb mean	1.00 a	1.00 a	1.26 b	1.22 b	4.32 d	2.90 c	4.98 e	4.93 e	2.89 b	2.51 a
Mar mean	1.34 a	1.24 a	2.62 c	2.13 b	4.20 b	3.21 d	5.00 f	4.96 f	3.29 b	2.88 a
General mean	1.16 b	1.08 a	2.32 d	1.93 c	4.39 f	3.33 e	4.97 h	4.79 g	3.21 b	2.78 a
	Šampion									
Dec 06	1.00 a	1.00 a	3.20 ij	1.90 def	4.93 pq	4.83 pq	4.74 opq	4.55 no	3.47 g	3.07 d
Feb 07	1.30 bc	1.18 abc	2.29 g	2.29 g	4.83 pq	2.98 hi	5.00 q	4.88 pq	3.35 efg	2.83 c
Mar 07	1.75 de	1.05 ab	2,29 g	1.70 d	4.70 op	3.80 lm	5.00 q	5.00 q	3.44 fg	2.88 c
Dec 07	1.03 a	1.00 a	4.04 m	1.98 ef	4.41 n	2.08 fg	5.00 q	5.00 q	3.62 h	2.51 a
Feb 08	1.43 c	1.00 a	1.00 a	1.00 a	5.00 q	3.10hi	5.00 q	5.00 q	3.11 d	3.52 a
Mar 08	1.00 a	1.00 a	3.20 ij	1.98 ef	3.80 lm	3.60 kl	5.00 q	5.00 q	3.26 e	2.89 c
Dec 08	1.00 a	1.00 a	4.00 m	2.00 ef	3.40 jk	3.08 hi	5.00 q	5.00 q	3.36 efg	2.76 bc
Feb 09	1.83 def	1.18 abc	2.33 g	2.27 g	4.80 opq	4.90 pq	5.00 q	5.00 q	3.49 g	3.33 ef
Mar 09	1.18 abc	1.18 abc	2.93 h	1.68 d	4.80 opq	2.98 hi	5.00 q	5.00 q	3.48 g	2.70 b
Dec mean	1.00 a	1.00 a	3.75 d	1.96 b	4.25 e	3.33 c	4.91 f	4.83 f	3.48 b	2.78 a
Feb mean	1.52 b	1.12 a	1.88 c	1.85 c	4.86 e	3.66 d	5.00 e	4.96 e	3.32 b	2.89 a
Mar mean	1.32 a	1.08 a	2.81 d	1.78 c	4.43 f	3.46 e	5.00 g	5.00 g	3.39 b	2.82 a
General mean	1.29 b	1.06 a	2.81 d	2.86 c	4.52 f	3.48 e	4.97 g	4.92 g	3.39 b	2.83 a

*Explanations: see Table 1

Table 3. Degree of damage to the shoots of three apple cultivars

	Golden Delicious									
	Field conditions		Freezing temperature (°C)						Mean	
			-20		-25		-30			
	Ctr	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA	Ctr	ALA
Dec 06	1.00 a	1.00 a	1.20 ab	1.55 cd	3.68 mn	3.20 ijkl	4.60 pq	4.35 p	2.63 de	2.53 d
Feb 07	1.00 a	1.00 a	1.83 de	1.75 de	5.00 r	3.35 jkl	5.00 r	5.00 r	3.21 ij	2.76 fg
Mar 07	1.00 a	1.00 a	2.20 fg	1.33 bc	5.00 r	3.95 no	5.00 r	5.00 r	3.30 j	2.81 fg
Dec 07	1.00 a	1.00 a	1.80 de	1.23 ab	4.03 o	3.40 klm	4.70 q	4.65 q	2.88 gh	2.57 d
Feb 08	1.00 a	1.00 a	1.58 cd	1.43 bc	3.78 no	2.47 h	4.55 pq	3.10 j	3.73 ef	2.00 b
Mar 08	1.00 a	1.00 a	3.43 klm	2.55 h	3.83 no	2.55 h	5.00 r	5.00 r	3.32 j	2.76 fg
Dec 08	1.00 a	1.00 a	3.15 ijk	2.45 gh	3.90 no	3.68 mn	4.55 pq	4.65 q	3.16 i	2.94 h
Feb 09	1.00 a	1.00 a	1.00 a	1.00 a	3.75 no	2.10 f	3.45 lm	2.48 h	2.31 c	1.64 a
Mar 09	1.00 a	1.00 a	3.44 klm	1.95 ef	4.48 pq	2.98 i	5.00 r	4.96 r	3.48 k	2.72 ef
Dec mean	1.00 a	1.00 a	2.05 c	1.74 b	3.87 e	3.42 d	4.62 f	4.55 e	2.89 a	2.72 a
Feb mean	1.00 a	1.00 a	1.48 b	1.39 b	4.18 e	2.64 c	4.33 e	3.51 d	2.75 b	2.13 a
Mar mean	1.00 a	1.00 a	3.02 c	1.94 b	4.43 d	3.16 c	5.00 e	4.95 e	3.37 b	2.76 a
General mean	1.00 a	1.00 a	2.18 c	1.69 c	4.16 e	3.07 d	4.65 g	4.34 f	3.00 b	2.53 a
	Gala									
Dec 06	1.00 a	1.00 a	1.10 ab	2.60 g	4.13 m	4.08 m	4.80 pg	4.88 pg	2.76 f	3.14 hi
Feb 07	1.00 a	1.00 a	1.00 a	1.00 a	2.73 ghi	2.88 hi	5.00 q	5.00 q	2.44 e	2.46 e
Mar 07	1.00 a	1.00 a	1.00 a	1.00 a	3.50 jk	3.68 kl	5.00 q	5.00 q	2.64 f	2.66 f
Dec 07	1.00 a	1.00 a	1.73 de	1.30 bc	4.90 q	3.38 j	5.00 q	5.00 q	3.16 i	2.66 f
Feb 08	1.00 a	1.00 a	1.00 a	1.00 a	3.83 l	2.98 i	5.00 q	3.46 jk	2.72 f	2.11 c
Mar 08	1.00 a	1.00 a	4.24 mn	3.35 j	3.83 l	3.60 jkl	5.00 q	5.00 q	3.52 j	3.22 i
Dec 08	1.00 a	1.00 a	1.43 c	1.30 bc	2.91 hi	1.68 d	4.63 op	2.95 hi	2.50 e	1.73 a
Feb 09	2.83 ghi	1.38 c	1.00 a	1.00 a	2.83 ghi	1.98 f	5.00 q	3.55 jk	2.92 g	1.97 b
Mar 09	2.73 ghi	1.38 c	2.70 gh	1.38 c	1.93 ef	1.98 f	4.78 pq	4.46 no	3.03 gh	2.30 d
Dec mean	1.00 a	1.00 a	1.42 b	1.73 c	3.98 e	3.04 d	4.81 g	4.26 f	2.81 b	2.51 a
Feb mean	1.62 b	1.12 a	1.00 a	1.00 a	3.13 d	2.61 c	5.00 f	3.99 e	2.69 b	2.18 a
Mar mean	1.59 b	1.12 a	2.66 d	1.91 c	3.08 e	3.08 e	5.00 f	4.95 f	3.08 b	2.77 a
General mean	1.41 b	1.08 a	1.70 d	1.55 c	3.40 f	2.91 e	4.91 h	4.35 g	2.85 b	2.47 a
	Šampion									
Dec 06	1.00 a	1.00 a	2.53 h	1.38 bc	3.94 m	1.98 efg	4.08 mno	4.36 op	2.89 de	2.18 a
Feb 07	1.23 ab	1.20 ab	1.98 efg	2.18 g	5.00 s	4.68 qr	5.00 s	5.00 s	3.30 g	3/25 g
Mar 07	1.00 a	1.00 a	2.80 hijk	1.98 efg	4.80 qrs	4.28 no	5.00 s	5.00 s	3.41 g	3.05 f
Dec 07	1.00 a	1.00 a	3.00 k	1.80 def	4.04 mno	3.35 l	5.00 s	5.00 s	3.27 g	3.78 d
Feb 08	2.63 hij	1.53 cd	1.00 a	1.00 a	4.80 qrs	3.58 l	5.00 s	3.96 mn	3.36 g	3.49 bc
Mar 08	2.03 fg	1.00 a	4.03 mn	2.58 hi	4.24 mno	2.86 ijk	5.00 s	4.08 mno	3.82 h	2.63 c
Dec 08	1.00 a	1.00 a	3.43 l	1.90 efg	4.04 mno	3.97 mn	5.00 s	5.00 s	3.37 g	2.96 ef
Feb 09	1.20 ab	1.15 ab	2.98 k	1.70 de	2.93 jk	2.10 fg	5.00 s	5.00 s	3.03 ef	2.48 b
Mar 09	2.03 fg	1.18 ab	4.12 mno	1.98 efg	4.60 pq	2.98 k	5.00 s	5.00 s	3.94 h	2.77 d
Dec mean	1.00 a	1.00 a	2.98 c	1.69 b	4.00 d	3.10 c	4.69 e	4.76 e	3.18 b	2.64 a
Feb mean	1.68 b	1.29 a	1.99 c	1.62 b	4.24 e	3.42 d	5.00 g	4.62 f	3.22 b	2.74 a
Mar mean	1.69 b	1.06 a	3.65 e	2.18 c	4.55 f	3.36 d	5.00 g	4.69 f	3.72 b	2.82 a
General mean	1.47 b	1.12 a	2.87 d	1.83 c	4.26 f	3.29 e	4.90 h	4.68 g	3.37 b	2.73 a

*Explanations: see Table 1

buds frozen at -20°C to the ALA treatment, buds treated with 5-ALA in the summer most frequently showed less damage (higher frost resistance) after being frozen at this temperature. This applies to the buds of all of the cultivars. Moreover, the difference in the scope of damage (as measured along the 5-point scale) between the treated and untreated buds increased along with the years of the experiment, which proves the cumulative influence of the treatment.

Freezing at -25°C always caused damage to the buds, and sometimes even totally destroyed them (Tab. 1.). However, except the first measurement date, the buds from trees treated with 5-ALA were less damaged than those from untreated trees, and the differences in damage were sometimes considerable, as for example, those observed in the buds of all cultivars in February 2008. The temperature of -30°C was most often too low and resulted in the total destruction of the buds regardless of whether 5-ALA had been applied or not.

The bud base, which contains a vascular bundle, is often more damaged by low temperatures than the buds. This can be observed in the spring when developing flowers dry out and fall. The reason is insufficient transport processes in the damaged tissues. The assessment of vascular bundles in bud bases taken directly from the orchard (field conditions) showed that their damage was small, and the positive effect of 5-ALA application was proven based on the measurement of frost damage to the 'Gala' bud bases on the last measurement date and to the 'Šampion' bud bases in March 2007 and February 2009 (Tab. 2.). 5-ALA treatment was found to have very strongly affected the frost resistance of the plant parts of all cultivars frozen at -20°C and -25°C. Similarly, as with the buds, 5-ALA treatment was found to have a negative influence only on 'Gala' bud bases frozen at -20°C in December 2006. But apart from that date, freezing at -25°C on all other dates showed a positive influence of 5-ALA treatment on the frost resistance of the bud bases of this cultivar. In addition, the 5-ALA treated bud bases of the other cultivars were significantly less damaged after freezing at -25°C, which is particularly apparent in the average damage for the same months of each year; except for the average damage for December of the 'Golden Delicious' bud bases, it was always significantly lower in plants treated with 5-ALA (Tab. 2).

The analysis of damage to shoots showed that shoots were the least sensitive to low temperatures of all of the examined plant parts (Tab. 3). In field conditions, damage was found only on 'Gala' shoots on the last two measurement dates and on 'Šampion' shoots in February of 2007 and 2008 and in March of 2008 and 2009. The 5-ALA applications limited the damage to shoots in each of the above periods. As with buds and bud bases, the treatment with 5-ALA proved to have a positive influence on the frost resistance of shoots frozen at -20°C and -25°C in most of the periods under analysis. Even if no difference in frost resistance was found between the treated and untreated shoots that were frozen at -20°C ('Golden Delicious' and 'Gala' in February), which was due to the small damage suffered by both treated and untreated shoots, those differences became apparent after freezing at lower temperatures (-25°C and -30°C).

The 'Gala' cultivar showed the greatest decrease in damage along with the years of the experiment: the response of all plant parts to 5-ALA application gradually changed from negative in December 2006 to positive on later measurement dates, when they were frozen at the lowest temperature (-30°C). The multi-annual average for February and March showed that the application of 5-ALA had a significant influence on the resistance of the plant parts of all of the apple cultivars (Tabs 1-3).

The assessment on the last date (March) in all of the years of the study showed the most positive influence of 5-ALA application on the frost resistance of buds, bud bases and shoots. The multi-year average scores obtained for damage showed that the 5-ALA treatment had a positive effect on all plant parts of each cultivar frozen at 20°C and -25°C. Irrespective of whether 5-ALA was applied or not, the temperature of -30°C was too low for the examined plant parts, except for the shoots of 'Golden Delicious'.

DISCUSSION

The application of 5-ALA had the smallest effect on samples frozen at the lowest temperature (-30°C), which entirely destroyed many of the examined plant parts regardless of the freezing date or cultivar. Some apple cultivars tolerate drops in temperature to as low as -47°C without damage (Hołubowicz 1984). Frost-sensitive cultivars such as 'Topaz', if grown in a favourable location, can tolerate temperatures as low as -36°C without damage (Zydlik and Zydlik 2008). The examined cultivars are sensitive to low temperatures and therefore already temperatures around -30°C may cause serious damage to them (Cline et al. 2012). Particularly valuable results were obtained on the last freezing date, i.e. in pre-spring, which is often critical for the growing of fruit trees because of damage occurring during this period (Hołubowicz 1984, Czynczyk et al. 2004).

An important regularity was observed for all of the examined cultivars. Namely, that 5-ALA application was more and more effective with each year of the research. This cumulative effect is probably attributable to the better nutritional status of the plants. A study conducted by Hotta et al. (1997) showed that low concentrations of 5-ALA had a variety of physiological effects for biosyntheses, such as plant growth, chlorophyll synthesis and photosynthesis. After a prolonged vegetation period, a plant enters the dormancy period later (Hołubowicz 1984). This was probably the reason behind the increased damage in December 2006. This explanation seems all the more plausible as no damage occurred in field conditions or during freezing on subsequent dates.

Damage seldom occurs in high winter since the resistance to frost is most often sufficient during this period (Hodun et al. 1999). At the end of January, the resistance of apple shoots to low temperatures considerably increases, which is certainly due to negative temperatures, which draw water from plant cells and increase sugar concentrations (Pukacki 2011). However, the subsequent stages in the process of building resistance are induced by external factors, such as the shortening photoperiod or a gradual fall in temperature (Hołubowicz 1984, Pukacki 2011). Due to these factors, plants may achieve maximum resistance earlier (if the temperature falls already in late autumn). In this research, differences in response to frost depended on cultivar and year. Freezing in December caused the smallest average damage to 'Golden Delicious' and the biggest average damage to 'Gala'. But the greatest average effect of treatment in all years of the research was observed in December for 'Šampion', since 5-ALA application reduced damage to all of the examined plant parts by 0.7 points. Most likely, the 5-ALA treatments contributed only negligibly to such great differences in response in the examined cultivars. The observed differences most certainly resulted from the time each of the cultivars entered the dormant period. 'Golden Delicious' flowers early and ends its vegetation early despite a late harvest period (Ugolik 1996). 5-ALA application to 'Gala', though hardly effective in December, yielded considerable results in high winter, i.e. in February. During this period of freezing, 5-ALA treatments had a more positive influence on 'Gala' than on the other cultivars – regardless of the freezing temperature and the examined plant part, the damage was reduced on average by 1.43 points.

Hotta et al. (1997) reported an increased level of abscisic acid (ABA) in rice leaves after ALA application. It is often stressed that this acid is an important factor increasing frost resistance, although the mechanism of this phenomenon is not well known (Pukacki 2011). Another physiological process that may have caused an increased resistance of the examined plant parts to frost damage is the change in the activity of amylase triggered by the RsBAMY1 specific protein, as described by Hara et al. (2010), whose concentration increases in 5-ALA-treated plants. The result is a decrease in the starch accumulated as an energy store and an increase in glucose. This starch-degrading enzyme enhances the soluble solid content, which decreases the temperature of ice formation in cells (Hołubowicz 1978). Additionally, the glucose is degraded through glycolysis and the citric acid cycle to produce ATP and nicotinamide adenine dinucleotide, which are energy sources for cells (Hara et al. 2010).

Conducted in Poland, the research may be of considerable relevance locally, as Poland is one of the world's largest apple producers (FAOSTAT 2015). It can also significantly contribute to the development of a method, or a set of methods, to effectively protect apple trees against frost. Native to temperate climate zones, apple trees are relatively frost-resistant; nevertheless, they do sometimes suffer from severe frost damage, as was the case in the winter of 1986/87 when the frost killed over 13 million apple trees (Palmer et al. 2003). Research on frost resistance is a valuable source of information about the size of damage or susceptibility to frost damage. It does not take account of the regenerative capabilities of plants, which depend on the species, cultivar or weather conditions in spring (Khorshidi et al. 2015), but a plant's capability to resist frost damage in different periods in winter, which certainly depends on its nutritional status (Watanabe et al. 2000, Łysiak and Kurlus 2009, Kurlus and Łysiak 2014). Appropriate frost damage prevention not only allows growers to achieve satisfactory crops, but also to protect the plants from the increased incidence of pathogen infections, which occur particularly easily on weakened plants (Schoeneweiss 1975).

However, the factors contributing to cold tolerance have not been clearly established. Further studies are needed to understand the physiological functions affected by 5-ALA, in particular, those involved in protection against cold stress.

CONCLUSIONS

1. Foliar ALA application reduces the damage of sensitive parts of apple trees resulting from low temperatures in winter.
2. ALA had a positive effect on frost resistance increases in subsequent years of ALA application.
3. The most positive effect of ALA application on frost resistance was observed on parts frozen on the last freezing dates in March, when the plants were at the end of their winter dormancy.

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AUTHOR CONTRIBUTIONS

G.P.Ł. developed the concept; G.P.Ł. and R.K. designed the experiments and collected data; G.P..Ł and A.M. analysed the data and wrote the paper.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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