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Full Length Research Paper

Seasonal alteration of sugar metabolism in strawberry (*Fragaria* × *ananassa*) plants during cold-acclimated and non-acclimated stages

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Plants of strawberry cvs Aromas and Diamante were removed from the field in cold acclimated (CA, January) and non-acclimated (NA, July) stages. Crown parts of the plant were used for analysis. Apoplastic total soluble sugar (TSS), reducing sugars and sucrose contents did not change in both cultivars in both sampling stages. Symplastic TSS, reducing sugars and sucrose contents were higher in CA stage than that in NA stage in all samples. Considering the cultivars, TSS, reducing sugars and sucrose contents were higher in Diamante than Aromas. The activity of acid invertase (EC 3.2.1.26) enzyme was higher in NA stage than that in CA stage in the apoplast of crown tissues. In general, acid invertase activity in the symplast of crown tissues in both cultivars was higher in CA stage compared with that in NA stage although not significantly, as a function of hardening. In addition generally, the cultivars had significantly higher sucrose synthase (SS) (EC 2.4.2.13) activity in the symplast of crown tissues when sampled at the CA than at the NA stage. In conclusion, cold-hardiness of strawberry was suggested by increasing their TSS, reducing sugars and sucrose contents significantly in the symplast of crown tissues in the CA stage. Moreover, both acid invertase and SS are regulated by cold-acclimation and deacclimation.

Key words: Acid invertase, apoplast, cold-acclimation, *Fragaria x ananassa*, soluble sugar, strawberry, sucrose synthase, symplast.

INTRODUCTION

Low temperature is one of the most important factors effecting plant development, distribution and productivity negatively. Many plants increase in freezing tolerance upon exposure to low nonfreezing temperatures, a phenomenon known as cold acclimation (Levitt, 1980; Thomashow, 1999). This process results in various biochemical changes include the expression of cold-stress proteins, such as dehydrins (Thomashow, 1999), the accumulation of sugars (Carpenter and Crowe, 1988) and sugar alcohols (Stitt and Hurry, 2002), changes in sugar metabolizing enzymes (Livingston and Henson, 1998) and in lipid composition (Uemura et al., 1995), and enhancements of antioxidative mechanisms (Thomashow, 1999).

In plant cells exposed to stresses, initial events come out mostly in apoplastic space (Atıcı and Nalbantoglu, 2003). Apoplast includes cell walls, air space, and vessel. It buffers the symplast from the severe abiotic and biotic environment (Sakurai, 1998). Freezing temperatures can cause extracellular ice formation, which lowers apoplastic water potential, dehydrates the symplast, and destabilizes cellular membranes (Steponkus, 1984).

During extracellular freezing, ice first forms in the dilute apoplastic solution and a water potential gradient established between the extracellular ice crystal and the intracellular liquid water (Levitt, 1980).

Many overwintering plants accumulate sugars, amino acids and antifreeze compounds including antifreeze

Abbreviations: CA, Cold-acclimated; **DDT,** DL-dithiothreitol; **DIECA,** diethyldithiocarbamic acid; **1PH,** first phase hardening; **FW,** fresh weight; **IWF,** intracellular washing fluid; **NA,** non-acclimated; **2PH,** second phase hardening; **SPS,** sucrose phosphate synthase; **SS,** sucrose synthase; **TSS,** total soluble sugar; **UDPG,** uridine 5' diphosphate glucose.

proteins in the apoplastic region (Livingston and Henson, 1998; Taşgın et al., 2003, 2006). Besides that Livingston and Henson (1998) have shown that cold stress is capable of inducing the synthesis of sugar metabolizing enzymes in the apoplastic space of crown tissue in winter oat.

Environmental stresses affect enzymes involved in both synthesis and cleavage of sucrose (Stitt and Hurry, 2002). Sucrose is degraded by either invertases or SS making a difference in the number of phosphorylable hexoses produced. Invertase hydrolysis produces glucose and fructose whereas SS cleavages produces uridine 5' diphosphate glucose (UDPG) and fructose, thus invertase action only amplifies the metabolic signal (Koch, 1996). The changes in activities of these enzymes at low temperature have been examined in wheat (Colderon and Pontis, 1985; Abdel-Latif, 2008), in spinach (Guy et al., 1992), in oat (Livingston and Henson, 1998), in cabbage (Sasaki et al., 2001) and in ryegrass (Bhowmik et al., 2006).

Strawberry (Fragaria x ananassa) is an economically important fruit species in Turkey and Turkey ranks second in the world after United States in strawberry production with 299.940 t (Anonymous, 2011). Strawberry is a semihardy evergreen and has relatively low tolerance to cold temperatures (Palonen and Buszard, 1997). However, strawberry cultivars vary widely in their cold hardiness, from 25 to -50°F (Barney et al., 1992). Several methods such as electrical conductivity (Harris, 1973), the recovery method (Harris, 1973), TTC reduction and tissue browning (Marini and Boyce, 1977) have been proposed for selecting cold-tolerant genotypes in strawberry. Some research also showed that strawberry exposed to low temperature can increase cold resistance through increase in activity of antioxidative enzymes (Gülen et al., 2008; Zhang et al., 2008). Besides that determination of sucrose, reducing sugars and total sugar differentiation has also been considered in cold-hardiness of genotypes in strawberry cultivars (Paquin et al., 1989). There is, however, no information available on the enzymes involved in sucrose metabolism in strawberry during cold-acclimation.

Both annual and perennial grown strawberry plants surviving during winter periods have been frequently exposed to low temperature and freezing stress during their different developmental periods. Therefore, resear-ches concentration on low temperature metabolism in strawbeery plant is very important. In this respect, the objective of this study was to understand how the plants survive freezing temperatures year-round; sugar and sucrose metabolizing enzyme activities were assayed in apoplastic extracts and symplastic tissues obtained from both cold-accli-mated and non-acclimated strawberry plants.

MATERIALS AND METHODS

Plant material

Uniform sized plants of approximately 1-year-old 'Aromas' and 'Diamante' cultivars were used. Both cultivars originally licensed by University of California. Thus, the two cultivars were adapted to different temperature zones. Turhan et al. (2011) recently determined the cell membrane injury of these cultivars. 'Diamante' was found to be relatively more tolerant to low temperatures than 'Aromas'. Six plants were removed at random from the field in Eskisehir, Turkey (39°47´N, 30°31´E) at CA (in January) and NA (in July) stages. Plants were packed on ice and brought to the laboratory. In January, the average temperature was 2.8°C (range -9.1 to 19.2°C). In July, the average temperature was 22.7°C (range 14.0 to 32.7°C). A linear relationship exists between the extent of damage to crowns and growth and/or productivity after cold injury (Bolduc and Paquin, 1987). Therefore, crown parts (the tissue remaining after removing leaves and roots) of strawberry (*Fragaria x ananassa*) plant were used for the analysis.

Preparation of intercellular washing fluid (IWF)

Apoplastic IWF was extracted from crown tissues according to Frecht-Christoffers et al. (2003). The tissues were cut into small pieces (*ca.* 5 mm in width) and rinsed for 2 min with tap water and immersed in distilled water for 1 min, then vacuum infiltrated for 5 min under a weak vacuum (-40 kPa). The infiltrated tissue was then surface dried gently and centrifuged at room temperature for 1 min at 1,200 g. The resulting water wash (apoplast) was collected in a plastic tube placed in liquid N_2 and kept at –80°C to await further analyses. 1 g of the tissue that remained after removal of the apoplast (that is, the symplast) was put into liquid N_2 and kept at -80°C to await further analysis.

Soluble sugars

To determine apoplastic sugars, a 100 µl aliquot was sampled from the apoplastic fluid. Symplastic sugars were extracted by suspending 0.1 g of crown pieces (after apoplast removal) in 5 ml of 80% (v/v) ethanol in an 85°C water bath for 1 h and then collecting the ethanolic liquid. This procedure was repeated four times for 1 h, 30 min, 15 min and 15 min. The ethanolic solutions were combined and evaporated to dryness at 55°C with the aid of continuous ventilation. The dried sugars were dissolved in 1 ml of distilled water and kept frozen at -20°C until determination. TSS and sucrose concentrations were determined by the anthrone reagent method, as modified for the determination of non-reducing sugars (Van Handel, 1968) by a spectrophotometer (Perkin Elmer Lambda 25, USA) at 620 nm using glucose and sucrose as the standards, respectively. Reducing sugar concentrations were determined colorimetrically with dinitrosalicylic acid (Miller, 1959) using glucose as the standard at 550 nm.

Sucrose metabolizing enzymes

Soluble (cytosolic) acid invertase activity in symplastic fraction of crown tissues was determined according to Aloni et al. (1991). In short, tissue samples of approximately 500 mg were ground in 5 ml ice-cold grinding medium containing 25 mM HEPES buffer (N2-2-ethanesulphonic acid) pH 7.2, 5 mM $MgCl_2$, 2 mM DDT (DL-Dithiothreitol) and 3 mM DIECA (diethyldithiocarbamic acid) as antioxidant. This mixture was centrifuged at 20 000 g for 20 min at 4°C. Aliquots of 100 µl of the supernatant were incubated in 10 ml 0.1 N phosphate citrate buffer pH 5.0 and 20 mM sucrose. The incubation was carried out for 30 min at 37°C and was terminated by addition of 1 ml dinitrosalicylic acid reagent. After boiling for 5 min, the resulting sugars were determined colorimetrically. SS activity was determined according to Aloni et al. (1996). Following

Table 1. TSS, reducing sugar and sucrose contents in the apoplast and symplast of in cold acclimated (CA, in January) and non-acclimated (NA, in July) crown tissues of strawberry cultivars.

Cultivar	Sample stage	TSS		Reducing sugar		Sucrose	
		Apoplast (mg ml^{-1})	Symplast (mg gFW^{-1})	Apoplast (mg ml^{-1})	Symplast (mg gFW^{-1})	Apoplast (mg ml^{-1})	Symplast (mg gFW^{-1})
Aromas	CA	46.4 ± 1.6[†]	52.5 ± 3.8	17.2 ±1.5	22.6 ± 1.3	15.3 ± 0.4	18.9 ± 0.6
	NA	50.4 ± 1.3	49.5 ± 3.8	18.3 ± 1.0	11.9 ± 1.6	16.1 ± 0.4	7.3 ± 0.4
Diamante	CA	46.1 ± 1.6	74.5 ± 3.8	19.8 ± 1.5	44.8 ± 1.6	15.3 ± 0.4	23.0 ± 0.6
	NA	44.7 ± 1.6	60.1 ± 3.8	20.4 ± 1.2	11.1 ± 1.3	16.2 ± 0.4	5.7 ± 0.4

[†] Data are expressed as means ± S.E. (n = 3).

extraction as described for acid invertase, the mixture was dialysed overnight in order to remove the internal sugars. The enzymatic activity was determined as sucrose breakdown on aliquots of 200 µl incubated in incubation medium containing 0.1 M phosphate-citrate buffer pH 7.0, 200 mM sucrose and 5 mM UDP. After incubation at 37°C for 30 min. the resulting fructose was determined by the dinitrosalicylic acid reaction. The data were expressed on fresh mass basis. Enzyme activities were expressed on a FW basis for the symplastic activity and on a volume basis for the apoplastic acid invertase activity. Total soluble protein contents of the crude enzyme extracts were determined according to Bradford (1976). Sucrose cleavage by sucrose synthase is only found in the cytoplasm (Dey and Harborne, 1997). Therefore, apoplastic SS activity was not determined.

Statistical analysis

The experiment was arranged in a randomized block design with three replications. Data were tested by SPSS 13.0 for Windows program and mean separation was accomplished by Duncan test at $P<0.05$.

RESULTS

Soluble sugars

As shown in Table 1, no significant effect was detected in TSS contents in the apoplast of crown tissues between the CA and NA stages. On the other hand, when data from the CA period were considered, TSS contents in the apoplast of crown tissues were almost the same in both cultivars (cv. Aromas: 46.4 mg/ml; cv. Diamante: 46.1 mg/ml). Two-way ANOVA did not reveal a significant effect of sampling stage, cultivars and the interaction of sampling stage and cultivars on TSS contents in the apoplast of crown tissues (Table 2).

Data from reducing sugar contents in the apoplast of crown tissues also did not indicate significant differences between CA and NA stages of both cultivars (Table 1). However, the highest reducing sugar contents in both CA and NA stages were detected in cv. Diamante. Two-way ANOVA did not reveal a significant effect of sampling stage, cultivars and the interaction of sampling stage and cultivars on reducing sugars in the apoplast of crown tissues (Table 2).

Generally, the cultivars had higher sucrose contents in the apoplast of crown tissues when sampled at NA stage than at the CA stage (Table 1). However, as with TSS and reducing sugar, two-way ANOVA did not reveal a significant effect of sampling stage, cultivars and the interaction of sampling stage and cultivars on sucrose contents in the apoplast of crown tissues (Table 2).

TSS contents in the symplast of crown tissues in CA and NA stages are shown in Table 1. TSS contents of both strawberry cultivars were significantly higher in CA stage than NA stage. TSS content was higher in cv. Diamante (74.5 mg/ g FW) than in cv. Aromas (52.5 mg/ g FW) at CA stage. Similarly, cv. Diamante (60.1 mg/ g FW) had higher TSS contents than cv. Aromas (49.5 mg/ g FW) in NA stage. Two-way ANOVA revealed a significant effect of sampling stage and cultivars but not the interaction of sampling stage and cultivars on TSS contents in the symplast of crown tissues (Table 2).

As shown in Table 1, reducing sugar content in the symplast of crown tissues in cold tolerant cv. Diamante was significantly higher in the CA stage compared with the NA stage. However, no significant difference was detected among reducing sugar contents of cultivars in NA stage (cv. Aromas: 11.9 mg/ g FW; cv. Diamante: 11.1 mg/ g FW). Two-way ANOVA revealed a significant effect of sampling stage, cultivars and the interaction of sampling stage and cultivars on reducing sugars in the symplast of crown tissues (Table 2).

Both cultivars had significantly higher sucrose contents when sampled at CA than at NA stage (Table 1). When data from CA period were considered, sucrose contents in the symplast of crown tissues of cv. Diamante were higher than those in cv. Aromas. There were no significant differences among cultivars in sucrose content in NA period. Two-way ANOVA revealed a significant effect of sampling stage, cultivars and the interaction of sampling stage and cultivars on sucrose content in the symplast of crown tissues (Table 2).

Table 2. Results of variance analysis (ANOVA) of stage (S), cultivar (C), and their interactions with TSS, reducing sugars content, sucrose content, acid invertase activity and sucrose synthase activity (SS) in apoplast and symplast of crown tissues in strawberry cultivars.

Dependent variable	Independent variable		
	S	C	S×C
TSS content in apoplast	0.652	3.612	3.035
TSS content in symplast	5.338*	18.813*	2.287
Reducing sugars content in apoplast	0.440	3.374	0.029
Reducing sugars content in symplast	223.899*	52.023*	60.592*
Sucrose content in apoplast	3.793	0.059	0.002
Sucrose content in symplast	798.489*	5.909*	30.741*
Acid invertase activity in apoplast	20.149*	5.670	3.086
Acid invertase activity in symplast	0.719	50.266*	0.197
SS activity in symplast	23.896*	1.036	1.217

*, Significant and non significant at $P < 0.05$. Numbers represent F values at 0.05 level.

Sucrose metabolizing enzymes

Acid invertase activity in the apoplast of crown tissues indicated significant differences between CA and NA stages in both cultivars (Figure 1). Acid invertase enzyme activity was significantly greater in NA stage than in CA stage in both cultivars. Acid invertase enzyme activity was higher in cv. Diamante than in cv. Aromas in CA stage. The highest acid invertase activity in NA stage was detected in cv. Diamante (~ 9.3 mg/ml prot./h) while the lowest activity was detected in cv. Aromas (~ 4.6 mg/ml prot./h). Two-way ANOVA revealed a significant effect of sampling stage but not the cultivars and the interaction of sampling stage and cultivars on acid invertase activity in the apoplast of crown tissues (Table 2).

In general, acid invertase activity in the symplast of crown tissues in both cultivars was higher in CA stage compared with those in NA stage although not significantly, as a function of hardening (Figure 2). When data from both sampling stages were considered, acid invertase activity was higher in cv. Diamante than in cv. Aromas. Two-way ANOVA revealed a significant effect of cultivars but not the sampling stage and the interaction of sampling stage and cultivars on acid invertase activity in the symplast of crown tissues (Table 2).

Generally, the cultivars had significantly higher SS activity in the symplast of crown tissues when sampled at the CA than at the NA stage (Figure 3). When data from the CA period were considered, it was determined that both cultivars had almost same SS activity in the symplast of crown tissues (~ 3.5 mg/g prot./min). Beside that, SS activity in the symplast of crown tissues was almost same in both cultivars (cv. Aromas: 2.8 mg/g prot./min; cv. Diamante: 2.4 mg/g prot./min) in NA period. Two-way ANOVA revealed a significant effect of sampling stage but not the cultivars and the interaction of sampling stage and cultivars on SS activity in the symplast of crown tissues (Table 2).

DISCUSSION

Soluble sugars

Sugars have been considered to be essential components in freezing tolerance. Sugars are known to lower the freezing point and increase the intracellular osmotic potential. This would reduce the amount of dehydration during extracellular freezing (Levitt, 1980). The apoplast is the first plant compartment encountering environmental signals (Gao et al., 2004). As shown in Table 1, there were no differences in TSS, reducing sugars and sucrose contents in the apoplast of crown tissues. Livingston and Henson (1998) also have found that a lack of change during first phase hardening (1PH) in the percentage of apoplastic carbohydrates in winter oat. The authors suggested that this is not a factor in the universally recognized increase in freezing tolerance of winter cereals during 1PH and other mechanisms must be responsible for this adaptation. Even though there were no differences in TSS, reducing sugars and sucrose contents in the apoplast of strawberry crown tissues (Table 1), an increase in apoplastic sugars in the crown of rye has been reported (Olien, 1984) and winter oat (Livingston and Henson, 1998) during second phase hardening (2PH). Olien (1984) also suggested that these sugars helped prevent adhesion of ice to critical cellular tissue during freezing. However, Canny (1995) pointed out that the solute concentration in the apoplast is not uniform.

The results indicate that during CA period, symplast of crown tissues had significantly higher TSS contents than during the NA period (Table 1), which paralleled their freezing tolerance. Increases in TSS ameliorate the impact of dehydration associated with freezing (Thomashow, 1999). Paquin et al. (1989) have showed that total sugars are related to cold hardiness clearly in strawberry crown. TSS contents were associated with

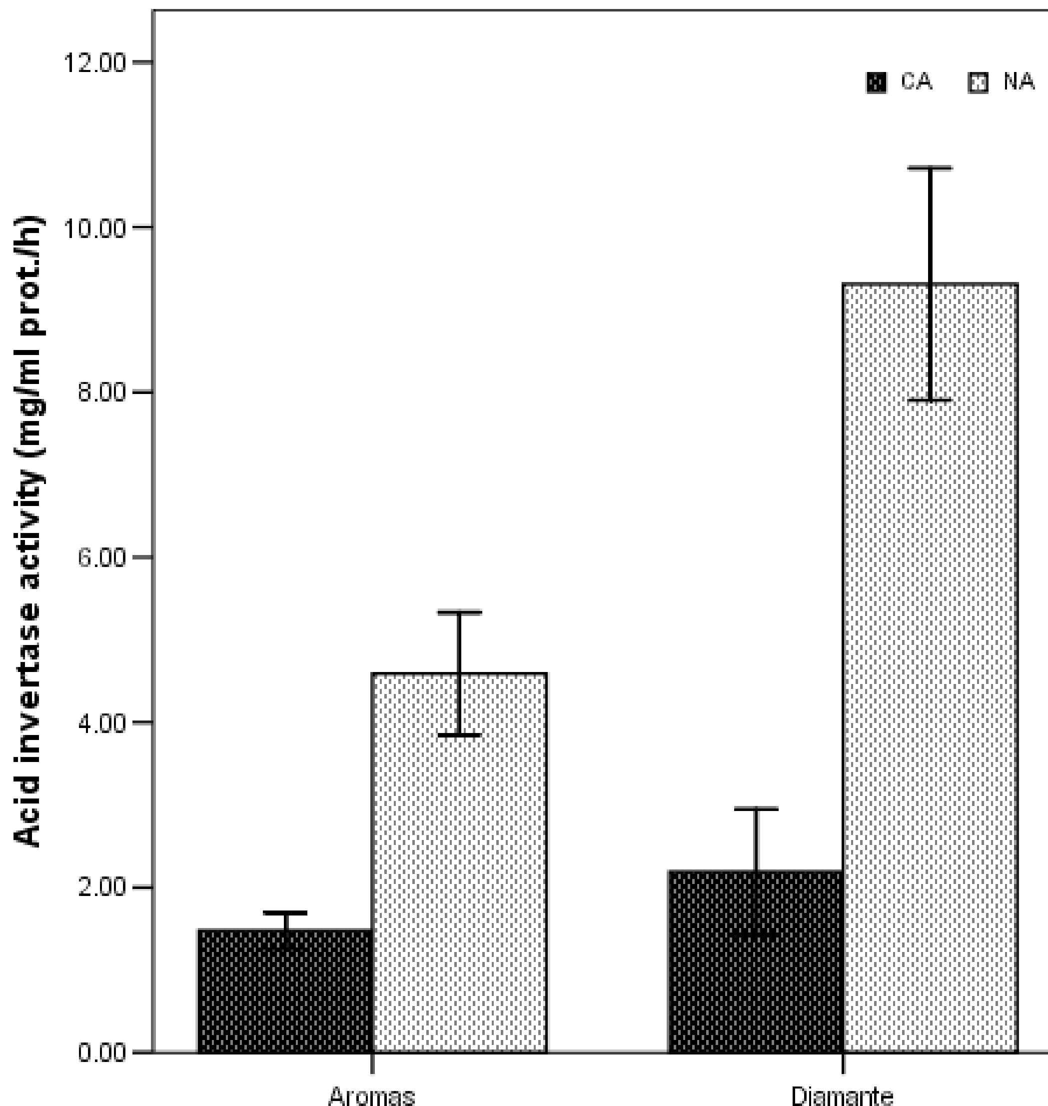

Figure 1. Apoplastic acid invertase activity in cold-acclimated (CA, in January) and non-acclimated (NA, in July) crown tissues of strawberry cultivars. Error bars represent ± SE of three replications.

increase in hardiness also in peach (Burak and Eris, 1992), raspberry (Palonen, 1999), olive (Eris et al., 2007; Gulen et al., 2009), cabbage (Sasaki et al., 1996, 2001) and wheat (Stupnikova et al., 2002).

In this study, reducing sugar and sucrose content were significantly greater in CA stage than in NA stage in the symplast of crown tissues in both cultivars (Table 1). Reducing sugar content was higher in cold-tolerant cv. Diamante compared with cv. Aromas in CA stages (Table 1). On the other hand, sucrose content was higher in cv. Aromas compared with cv. Diamante in CA stages (Table 1). There is no information about symplastic sugar contents during cold acclimation. On the other hand, besides total sugars, reducing sugars and sucrose have also been reported in the crowns of Redcoat and Bounty straw-berries related to cold hardening (Paquin et al., 1989). As early as 1965, Trunova (Livingston and Henson, 1998) found that after 2 PH found decreased levels of fructan and increased levels of glucose, fructose, and sucrose in whole crown tissue of wheat. He suggested that the sugar increase during 2PH was providing osmotic protection to cells in the over-wintering organ (crown) of the plant (Livingston and Henson, 1998).

The most rapidly accumulated soluble sugar in res-ponse to low temperature is sucrose (Guy et al., 1992). However, sugar accumulation at low temperature is not limited to only sucrose. The soluble carbohydrates, sucrose, a high ratio of sucrose to glucose plus fructose in raspberry were correlated with cold resistance (Palonen, 1999). TSS, particularly glucose and sucrose contents were also related to freezing tolerance clearly in

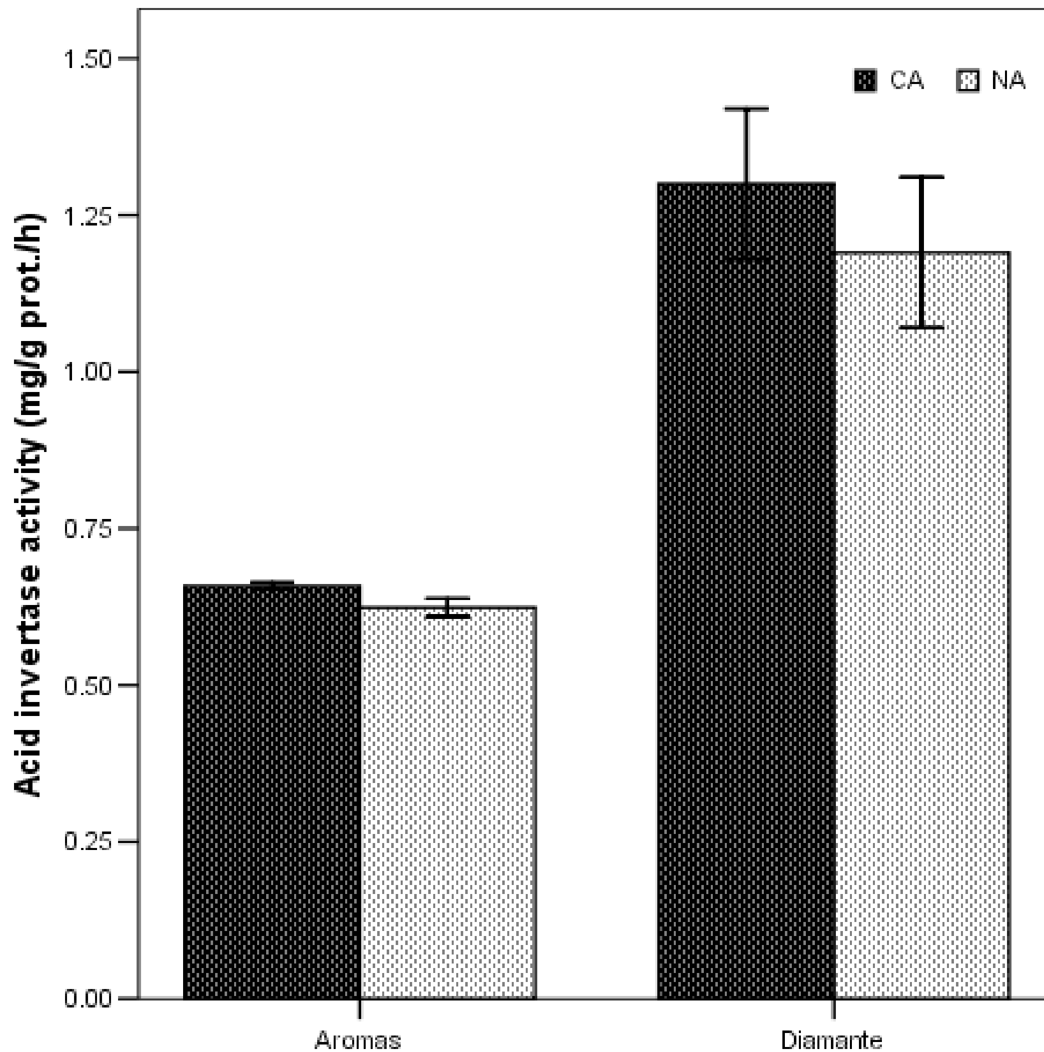

Figure 2. Symplastic acid invertase activity in cold-acclimated (CA, in January) and non-acclimated (NA, in July) crown tissues of strawberry cultivars. Error bars represent ± SE of three replications.

olive (Eris et al., 2007; Gulen et al., 2009).

Sucrose metabolizing enzymes

Invertase, sucrose phosphate synthase (SPS) and SS are directly involved in sucrose synthesis and/or degradation (Sasaki et al., 2001). In plants, sucrose is cleaved by either invertase or sucrose synthase. Invertase activity is found in the cytoplasm, vacuole, and apoplast, whereas sucrose cleavage by sucrose synthase is only found in the cytoplasm (Dey and Harborne, 1997). However, alteration in activities of these enzymes at low temperature varied between the plant species. In the present study, acid invertase activity in the apoplast of crown tissues was significantly higher in NA stage than in CA stage in both cultivars (Figure 1). Although invertase activities in the apoplastic fluid from crowns were not different during non hardening and 1PH winter oat plants, it increased in the apoplast from crowns of 2PH plants (Livingston and Henson, 1998).

In general, acid invertase activity in the symplast of crown tissues in both cultivars was higher in CA stage compared with those in NA stage although not significantly, as a function of hardening (Figure 2). On the other hand generally, the cultivars had significantly higher SS activity in the symplast of crown tissues when sampled at the CA than at the NA stage (Figure 3).

There is no information about symplastic sugar metabolizing enzymes during cold acclimation. However, similarly invertase activities in whole crown tissue increased slightly, although not significantly, as a function of hardening in winter oat (Livingston and Henson, 1998). Calderon and Pontis (1985) also reported that the activity of SS rose continuously, immediately after the chilling shock in wheat. In addition, it was founded that acid

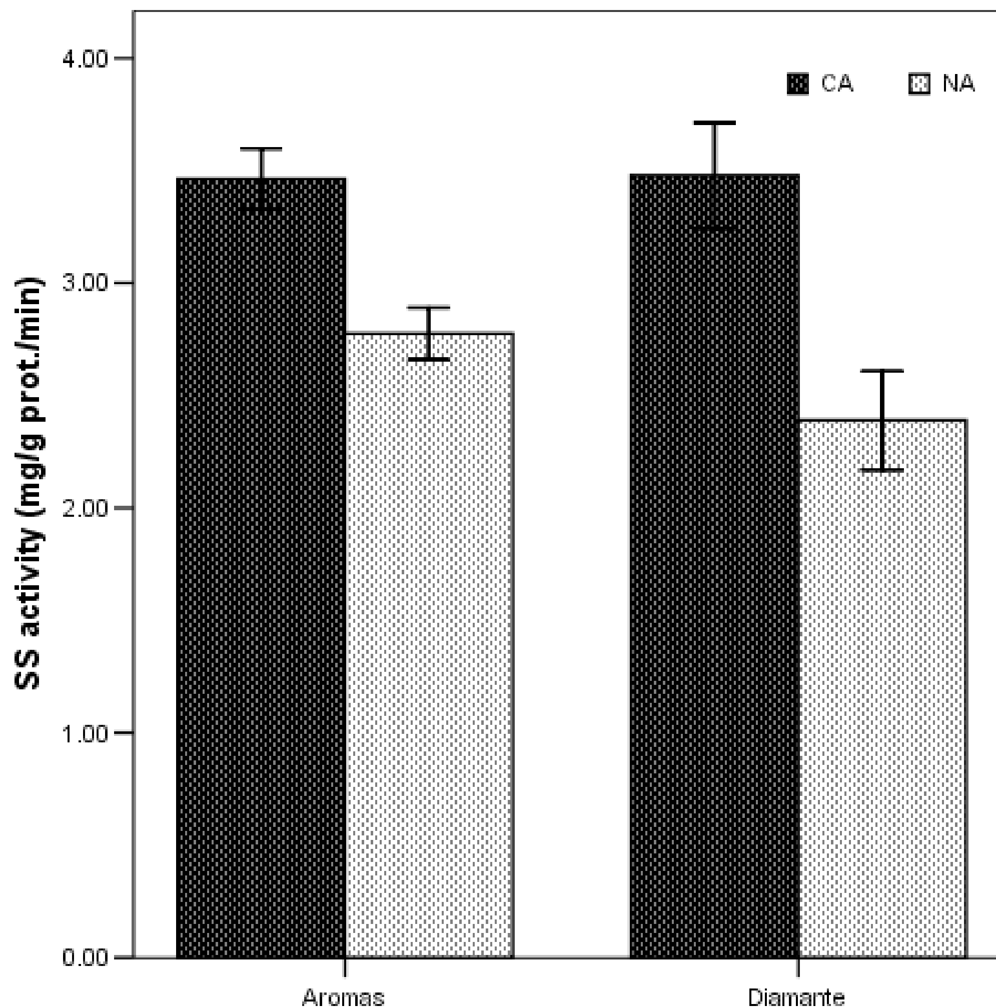

Figure 3. Symplastic SS activity in cold-acclimated (CA, in January) and non-acclimated (NA, in July) crown tissues of strawberry cultivars. Error bars represent ± SE of three replications.

invertase, SS and SPS are regulated by cold acclimation and play an important role in sugar accumulation and acquisition of freezing tolerance in ryegrass (Bhowmik et al., 2006). On the other hand, Sasaki et al. (2001) suggest that SS and SPS, but not acid invertase, are regulated by cold-acclimation and deacclimation and play important roles in sugar accumulation and acquisition of freezing tolerance in the leaves of cabbage seedlings.

Consequently, seasonal changes in TSS, reducing sugars and sucrose contents were related to changes in cold-hardiness and air temperatures in strawberry plants. In addition, both acid invertase and SS are regulated by cold-acclimation and deacclimation in strawberry plants.

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