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Antitranspirant treatment on bean plants to counteract cold stress

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Abstract: The aim of the work was to test the efficacy of the antitranspirant Scudotherm® in preventing cold damages on *Phaseolus vulgaris* L. Two tests were conducted: the first between June-July 2018, and the second between May-June 2019. Plants were grown in a greenhouse, in plastic pots (10 pots/treatment), on a peaty substrate. Treatments were carried out on three-week-old plants, by foliar application 24 hours before the stress. The experimental design included treatments with Scudotherm® at 2% compared to an untreated and unstressed control, and to an untreated but stressed control (controls were sprayed with tap water). The cold stress was induced by placing the plants at 3-4 °C, for 48 hours. Both *in vivo* and destructive analyzes were performed to evaluate the health status of plants, immediately after stress and after a one-week recovery period. The analyzes were performed on three biological replications. No significant effect of the treatments was observed on chlorophylls *in vivo*, chlorophyll *a* fluorescence parameters, secondary metabolites (anthocyanins and phenolic index), and lipid peroxidation. Significant variations were found in the metabolism of the sugars. Scudotherm® allowed maintaining the sucrose concentration similar to control and lower than stressed plants. During the first year, a similar behavior was also recorded for total sugars. These results suggested that Scudotherm® is able to mitigate some of the negative consequences of low temperatures, acting as a physical barrier on leaves, with an indirect physiological and biochemical effect.

Keywords: *Phaseolus vulgaris* L., abiotic stress, Scudotherm®, low temperatures, sugars.

1. Introduction

Among the major limiting factors in agriculture, sub-optimal temperature certainly occupies a pivotal position. Non-freezing temperatures, ranging from 0 to 10 °C, could represent a chilling stress condition for many macrothermal crops and may cause substantial produce loss during the early growing season (El-Saht, 1998; Allen and Ort, 2001; Piasecka et al., 2019). Species originating from tropical and subtropical regions are much more susceptible to this abiotic stress (McWilliam et al., 1982; Badowiec and Weidner, 2014). In chilling sensitive crops, the exposure to low temperatures, below 10-12 °C, can induce a reduced metabolism and delayed physiological responses, up to an arrest of vegetative growth. At cellular level, the cell membrane can be injured with disruption of the phospholipid double layer (Galindo et al., 2007; Yadav, 2010). It is possible to observe a reduction of the photosynthesis and an increase of respiration and healing reactions. Severe damage can rapidly lead to cell death and leaf desiccation. Chilling injury can be firstly observed in younger leaves of plants. Cold stress at non-lethal temperature induces the accumulation of reactive oxygen species and the increase of detoxification related enzymes. Melon plants exposed to 10 °C showed an accumulation of hydrogen peroxide along

with the increase of superoxide dismutase (SOD) and decrease of catalase (Rivero et al., 2002). Poor seed germination, stunted seedlings, necrotic lesions, leaf senescence/wilting, and tissues death can also be included among the several phenotypic symptoms observable after cold stress exposure (Hussain et al., 2018; Toscano et al., 2019). Moreover, plants tend to accumulate soluble sugars and other osmolytes to promote osmotic adjustment, enhancing the freezing tolerance. The concentration of several phenolic compounds is also enhanced in response to abiotic stresses, and sometimes this condition may enhance the nutritional value of the produce (Yoon et al., 2016; Piasecka et al., 2019; Toscano et al., 2019). In winter sweet spinach, the exposure to low temperatures increased the level of antioxidant compounds, thereby improving antioxidant activity. Regarding the effect on sugars, the sucrose concentration was increased by cold (Watanabe and Ayugase, 2015). Chilling symptoms typically increase with the time of exposure and with the temperature reduction, and they are often visible only after a period of exposure to non-chilling temperatures (El-Saht, 1998), defined as recovery period.

In some respects, it is possible to compare cold stress to drought. In fact, low temperatures may also cause turgor stress at the cellular level, inducing a real water deficit (McWilliam et al., 1982; Beck et al., 2007). Moreover, some of the physiological and molecular mechanisms related to water stress and cold tolerance are common in several ways (Lanna et al., 2018 and references therein). For this reason, the use of antitranspirant in agriculture, also in the case of cold stress, could be useful considering that these products are able to regulate/slow down leaf transpiration, protecting plants by coating the foliage. On the basis of their mode of action, antitranspirants can be classified into three major categories: physical antitranspirants, physiological antitranspirants and reflecting (Shinohara and Leskovar, 2014; Park et al., 2016 and references therein). The first group includes resins, waxes, latexes, gel or polymers that coat leaf surface and reduce water losses by blocking stomata (Goreta et al., 2007). On the other hand, physiological antitranspirants, such as abscisic acid (ABA), minimize the transpiration rate by inducing stomata closure in plants through metabolic processes. The third category includes products as kaolin clay and chitosan that reduce leaf temperature by increasing leaf reflectivity and determine a lower transpiration rate and a higher water use efficiency (Shinohara and Leskovar, 2014). Physical antitranspirants were shown to have positive effects, for example, in several herbaceous plants (Anderson and Kreith, 1978), in bedding plants (Park et al., 2016) and in pepper (*Capsicum annuum* L.) (del Amor et al., 2010; Jufri and Sulistyono, 2016). Generally speaking, film-forming antitranspirants are widely used in horticulture for a large number of crops.

The aim of this research was to study the effectiveness of a physical antitranspirant, Scudotherm®, in counteracting and alleviating the damages deriving from cold stress in bean plants. The common bean (*Phaseolus vulgaris* L.) is particularly sensitive to low temperatures. This legume is the most important for human consumption worldwide, especially in Latin America and in Africa (Beebe et al., 2009), and its cultivation suffers significantly of abiotic stress conditions, including drought, heat, chilling, waterlogging, salinity, and mineral toxicities. The hypothesis of this work was that, under chilling exposure, the application of an antitranspirant can provide a physical barrier to the excessive transpiration and, therefore, induce a reduction of water losses.

2. Materials and Methods

2.1. Plant material and treatments

Two trials were conducted during the research: the first between June and July 2018, and the second between May and June 2019. The three-week-old bean plants (*Phaseolus vulgaris* L., cv. Borlotto Lingua di Fuoco Nano sel. SEM) were grown in a glasshouse at the Faculty of Agricultural and Food Sciences of the University of Milan, in plastic pots (22 cm diameter) containing a peat-based substrate. The experimental design included the following three treatments: (a) plants treated with an antitraspirant and then exposed to cold stress, (b) an untreated and unstressed control, and (c) an untreated but

cold-stressed control. The foliar application (spray up to run off) of the antitranspirant Scudotherm® (an organic degradable copolymer, 45%, in liquid form, at a concentration of 2%; Agridaeus Srl., Magenta, Milano, Italy) was carried out 24 hours before plants were exposed to the cold stress. All the controls were sprayed only with tap water. The cold stress was imposed by placing the plants in a cold room at 4 °C (in 2018) or 3 °C (in 2019) for 48 hours. Each treatment was composed by ten plants randomly distributed on the greenhouse bench. To assess plant health, both *in vivo* and destructive analyzes were carried out. All the analyzes were performed on three biological replications. The 2018 trial ended immediately after the stress, whereas, in 2019, the trial also included a one-week post-stress recovery period of the plants, to assess their resilience.

2.2. Analyzes

2.2.1. Chlorophyll measurements *in vivo*

The chlorophyll content of leaves was estimated *in vivo* with a chlorophyll meter (CL-01, Hansatech, United Kingdom). This device provides an indication of green color and it determines relative chlorophyll content using dual wavelength optical absorbance (620 and 940 nm wavelength). The results were expressed in arbitrary units (a.u.).

2.2.2. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured using a hand-portable fluorometer (Handy PEA, Hansatech, King's Lynn, United Kingdom). Bean leaves were dark-adapted for 30 minutes. Using a leaf clip (4 mm diameter), a rapid pulse of high-intensity light of 3000 μmol/m^2/s (600 W/m^2) was administered to the leaf, inducing fluorescence. The fluorescence parameters were calculated automatically by the used device. In particular, we focused our attention on the Performance Index (PI) and the maximum quantum efficiency of PSII (Fv/Fm) related data.

2.2.3. Anthocyanins and phenolic index

For anthocyanins and phenolic index determination, fresh leaf tissue (30–50 mg) was extracted into methanolic HCl (1%), for 24 h at 4 °C in a dark room; afterward quantitative determination was carried out with spectrophotometer readingsat 320 nm (phenolic index) and 535 nm (anthocyanins) (Klein and Hagen, 1961).

2.2.4. Sugars determination

For all the sugars determinations, 1 g of leaf tissue was homogenized in 3 mL of distilled water and centrifuged at 3000 × g (ALC centrifuge-model PK130R) for 15 min at room temperature (RT). Sucrose was assayed according to the resorcinol method. Briefly, 0.2 mL of extract was added to 0.2 mL NaOH 2N and incubated at 100 °C for 10 min; then 1.5 mL of hot resorcinol solution was added and the sample was incubated at 80 °C for 10 min. The resorcinol solution was prepared by adding 35 mg of resorcinol and 90 mg of thiourea in 250 mL HCl 30%, mixed with 25 mL of acetic acid and 10 mL of distilled water. Samples were cooled at RT and spectrophotometer readings were performed at 500 nm (Rorem et al., 1960). Sucrose levels were calculated referring to a sucrose calibration curve [0, 0.5, 1, 1.5, and 2 mM] with a R^2 value of 0.9977. The analysis of reducing sugars was performed using 0.2 mL of crude extract that was added to 0.2 mL of a solution containing 62.6 mM dinitrosalicylic acid and 1.52 M potassium sodium tartrate. The reaction mixture was heated at 100 °C for 5 min, then 1.5 mL of distilled water was added and absorbance readings were performed at 530 nm (Miller, 1959). The reducing sugars concentration was expressed as glucose equivalent using a glucose standard curve [0, 1, 2, 3, and 4 mM] with a R^2 value of 0.9878. Total sugars were assayed according to the anthrone assay. Anthrone (0.2 g) was melted in 100 mL of H_2SO_4 and shook for 30–40 min. Then, 1 mL of the leaf tissue extract was added to 5 mL of anthrone solution, cooled in ice for 5 min and mixed thoroughly.

Samples were incubated at 95 °C for 5 min and then cooled on ice (Yemm and Willis, 1954). Absorbance was read at 620 nm and the levels were calculated referring to a glucose calibration curve [0, 0.25, 0.5, 0.75 and 1 mM] with a R^2 value of 0.9865.

2.2.5. Lipid peroxidation determination

The level of lipid peroxidation was measured with the thiobarbituric acid reactive substances (TBARS) assay (Heath and Packer, 1968). Around 1 g of fresh leaves was homogenized in 5 mL 0.1% trichloroacetic acid (TCA) solution. The extract was mixed with 4 mL of 20% (W/V) TCA, 25 µL of 0.5% thiobarbituric acid (TBA) and distilled water. After vortexing, the mixture was heated at 95 °C for 30 minutes in a water bath and then cooled on ice. Absorbance at 600 nm was subtracted from the absorbance at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS was expressed on the basis of fresh weight as malondialdehyde (MDA) equivalents (nmol/g FW), calculated using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1}.

2.2.6. Statistical analysis

The statistical analysis was performed with GraphPad Prism 6. All data were subjected to one-way ANOVA and differences among the treatment means were assessed by the Bonferroni post-hoc test. Each treatment was composed by ten plants randomly distributed on the bench. The analyses were performed on three biological replications. Additional information is reported in the figure legends.

3. Results

3.1. First experimental year

3.1.1. Chlorophyll measurements *in vivo,* anthocyanins and phenolic index

The chlorophylls measured *in vivo* in bean leaves showed similar values (around 7.6 a. u.) in all the treatments (Table 1). Secondary metabolites, determined in leaves, did not vary among treatments (Table 1). Anthocyanin concentrations ranged from 19.07 to 23.35 mg/100 g FW. Total phenols, expressed as phenolic index, showed a maximum values of 61.73 ABS_{320nm}/g FW (Table 1).

Table 1. Chlorophyll level, measured *in vivo* in bean leaves, and concentrations of anthocyanins and phenolic index measured in bean plants of the following treatments: unstressed control, stressed control (chilling stress), Scudotherm (Scudotherm application + chilling stress). Values are means (n=3) ± standard error of the means. Within each column, different letters indicate significant differences among treatments according to the Bonferroni post-hoc test ($P < 0.05$).

Treatment	Chlorophylls (arbitrary units)	Anthocyanins (mg/100 g FW)	Phenolic index (ABS_{320nm}/g)
Unstressed control	7.53 ± 0.77	23.35 ± 3.95	61.47 ± 3.11
Stressed control	7.70 ± 0.66	21.10 ± 4.34	55.18 ± 8.93
Scudotherm	7.67 ± 0.70	19.07 ± 4.10	61.73 ± 18.67

3.1.2. Sucrose, reducing sugars, and total sugars concentration

Sucrose levels did not vary among treatments (Figure 1A). As expected, the concentrations of reducing and total sugars (Figures 1B and 1C) were lower in unstressed control plants than in the other treatments. Chilling stress induced a significant increase in the concentration of reducing and total sug-

ars compared to the unstressed control (Figures 1B and 1C). Reducing sugars increased in both stressed control and in the Scudotherm treatment, showing double concentrations, around 9 g/kg FW in average, compared to control (Figure 1B). Total sugars showed the highest concentration in stressed control; it is interesting to observe that the Scudotherm® treatment allowed maintaining intermediate levels of total sugars (Figure 1C).

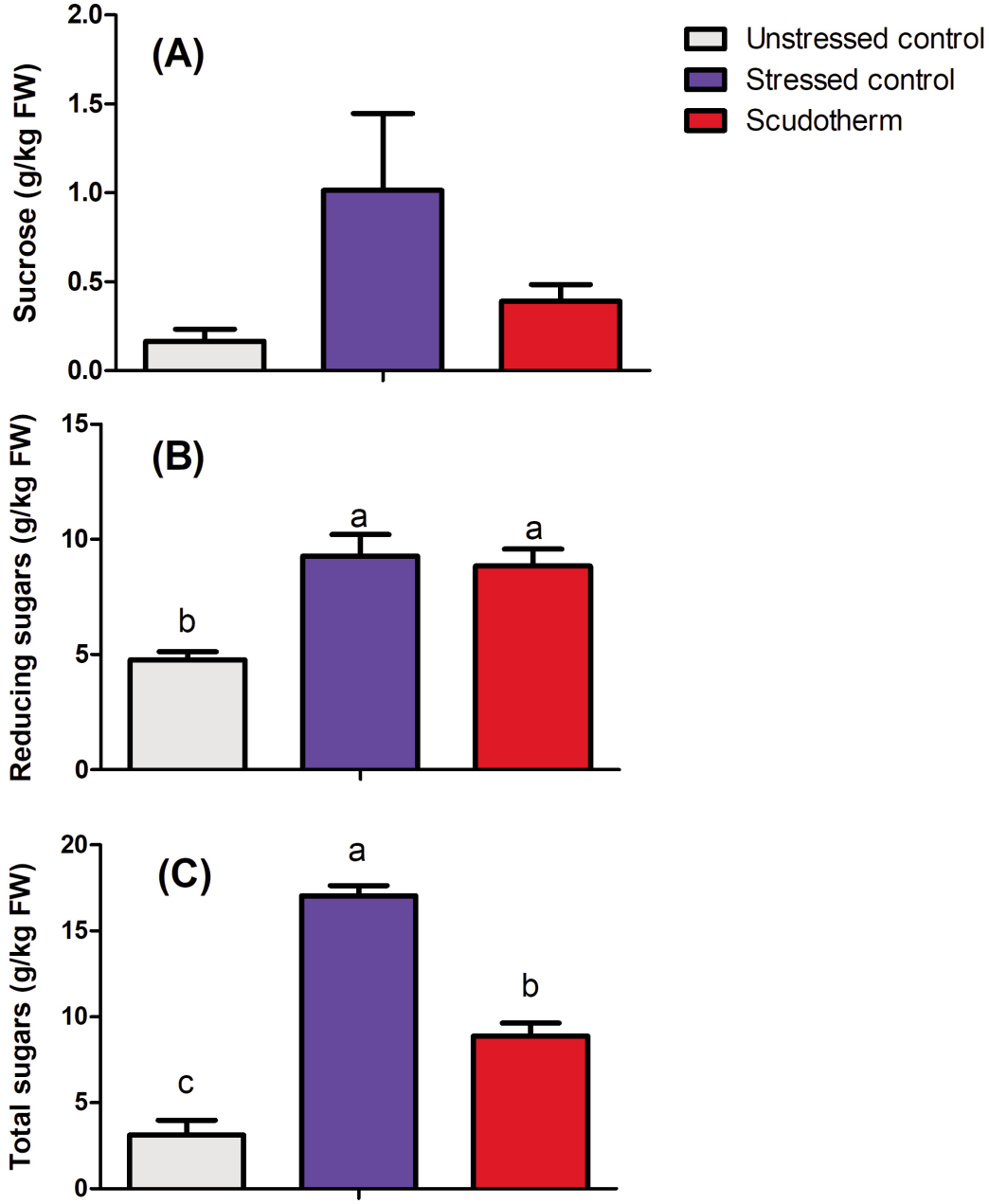

Figure 1. Sucrose (A), reducing sugars (B), and total sugars (C) measured in leaves of bean plants of the following treatments: unstressed control, stressed control (chilling stress), Scudotherm (Scudotherm application + chilling stress). Values are means (n=3) ± standard error of the means. In each panel, different letters indicate significant differences between treatments according to the Bonferroni post-hoc test ($P < 0.05$).

3.1.3. Lipid peroxidation

The level of lipid peroxidation, measured using the thiobarbituric acid reactive substances (TBARS) assay, was not affected by the treatments. The values ranged between 8.05 and 15.81 nmol/g FW.

3.2. Second experimental year

3.2.1. Chlorophyll measurements *in vivo* and chlorophyll *a* fluorescence

The second year of experiment was a repetition of the first trial but additional measurements were carried out in order to better understand the mode of action of the antitranspirant. Chlorophylls measured immediately after the chilling stress did not vary among treatments (Table 2). Generally speaking, after one week of recovery, chlorophylls tended to decrease; however, even at this time point, there were no significant differences among treatments. The maximum quantum efficiency of PSII (Fv/Fm) and the Performance Index (PI) did not show differences both immediately after the chilling stress and after one week of recovery (Table 2).

Table 2. Chlorophylls level, maximum quantum efficiency of PSII (Fv/Fm), and performance index (PI) measured *in vivo*, immediately after the stress and after one week of recovery, in leaves of bean plants of the following treatments: unstressed control, stressed control (chilling stress), Scudotherm (Scudotherm application + chilling stress). Values are means (n=3) ± standard error of the means. For all the parameters, no significant differences among treatments were detected according to the Bonferroni post-hoc test ($P < 0.05$).

Treatment	Post stress			Recovery		
	Chlorophylls (a.u.)*	Fv/Fm	PI	Chlorophylls (a.u.)*	Fv/Fm	PI
Unstressed control	8.45±0.60	0.85±0.01	2.46±0.11	7.31±0.90	0.83±0.01	2.90±0.65
Stressed control	10.96±1.51	0.84±0.01	2.58±0.17	6.64±1.44	0.81±0.01	1.33±0.25
Scudotherm	9.82±0.90	0.80±0.03	1.81±0.50	6.76±0.91	0.80±0.01	0.92±0.33

* a.u. = arbitrary units

3.2.2. Anthocyanins and phenolic index

Immediately after the cold stress, plants of the three treatments had similar values of anthocyanins (17 mg/100 g FW) (Table 3). During the recovery period, anthocyanins ranged between 9.15 and 16.62 mg/100 g FW, but no significant differences were observed among treatments. Differences among treatments were not significant also for the phenolic index, measured in bean leaves, because of the high variability observed (Table 3).

Table 3. Concentrations of anthocyanins and phenolic index, measured, immediately after the stress and after one week of recovery, in leaves of bean plants of the following treatments: unstressed control, stressed control (chilling stress), Scudotherm (Scudotherm application + chilling stress). Values are means (n=3) ± standard error of the means. For all the parameters, no significant differences among treatments were detected according to the Bonferroni post-hoc test ($P < 0.05$).

Treatment	Post stress		Recovery	
	Anthocyanins (mg/100 g FW)	Phenolic index (ABS 320nm/g)	Anthocyanins (mg/100 g FW)	Phenolic index (ABS 320nm/g)
Unstressed control	17.83±2.48	57.30±11.30	16.08±4.65	58.62±12.10
Stressed control	17.02±0.36	34.15±2.03	16.62±0.65	71.10±2.24
Scudotherm	17.12±0.82	36.00±0.95	9.15±1.11	72.02±3.70

3.2.3. Sucrose, reducing sugars, and total sugars concentration

Measurements related to sucrose and reducing sugars are reported in Figure 2 and confirmed the results observed for the first year in the determination performed immediately after the exposure to cold stress. Sucrose levels, after the stress, were lower in unstressed control plants compared to the other treatments. As expected, the highest sucrose concentration was observed in stressed control plants, while Scudotherm® treated plants showed an intermediate sucrose level (Figure 2A). During the recovery (Figure 2B), differences among treatments were not significant, indicating an effective recovery by the plants. Reducing sugars in post-stress and in recovery did not show significant variations among treatments (Figures 2C and 2D).

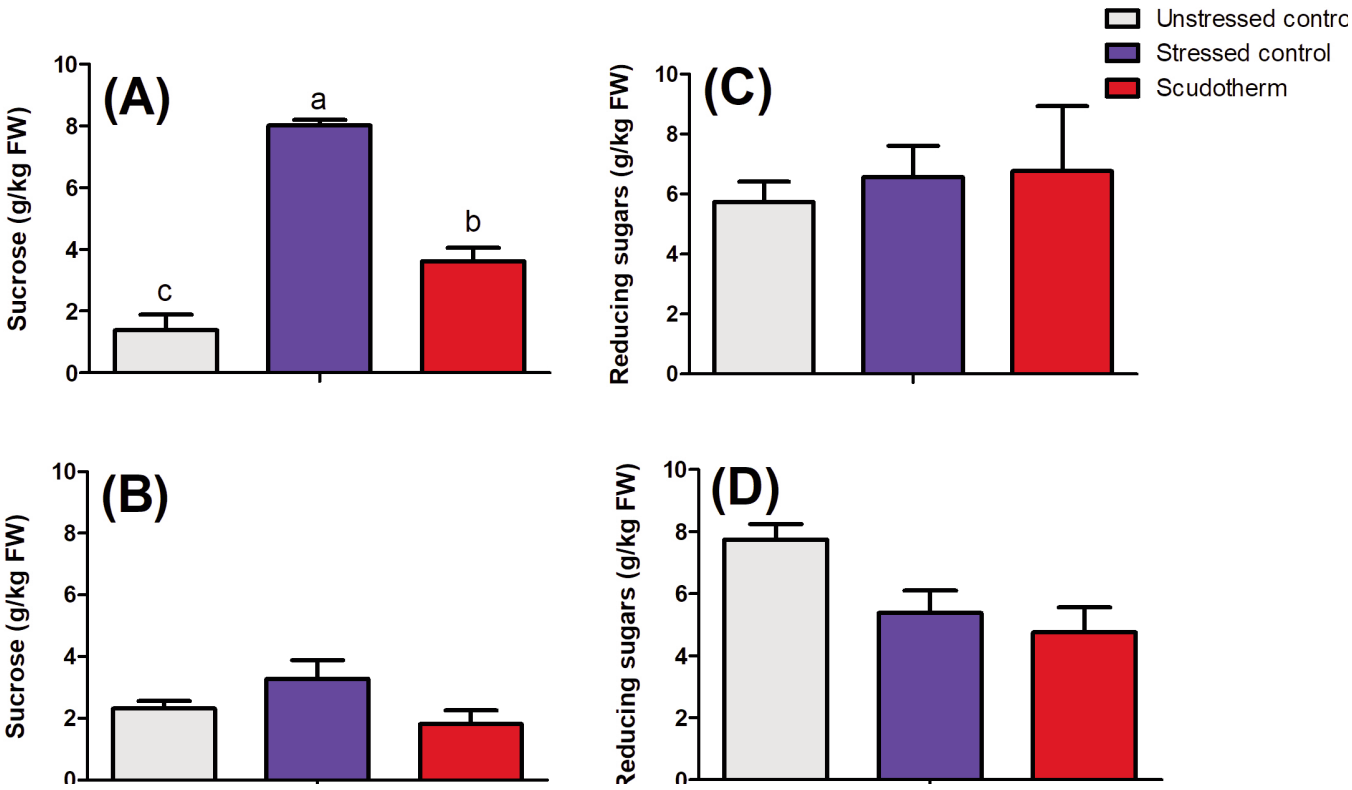

Figure 2. Sucrose and reducing sugars concentrations measured, immediately after the stress (A and C) and after one week of recovery (B and D), in leaves of bean plants of the following treatments: unstressed control, stressed control (chilling stress), Scudotherm (Scudotherm application + chilling stress). Values are means (n=3) ± standard error of the means. In each panel, different letters indicate significant differences among treatments according to the Bonferroni post-hoc test ($P < 0.05$).

3.2.4. Lipid peroxidation

The levels of lipid peroxidation did not differ among treatments, both immediately after the stress and after the recovery period. In the first sampling, values ranged between 5.87 and 11.10 nmol/g FW; after one week of recovery, the levels did not exceed 7.52 nmol/g FW.

3.2.5. Visual observation of damages

Immediately after the cold stress exposure, it was possible to observe that stressed control leaves showed the most evident damages (Figure 3). Scudotherm® treatment allowed to a much more moderate injure and only few damages were visible on leaf blade margins. As expected, unstressed control plants had no visible damages on leaf tissues.

Figure 3. Visual observation of damages on bean leaves immediately after the cold stress. Three representative leaves were selected: CS=stressed control, CNS=unstressed control, 3= Scudotherm (Scudotherm application + chilling stress).

4. Discussion

The purpose of the present work was to evaluate the efficacy of the antitranspirant Scudotherm® in preventing and counteracting cold stress on bean plants. Results suggest that Scudotherm® exerts a protective function to cold, acting as a physical barrier on leaves. This result is in line with the nature/type of the product used (physical antitranspirant) in our trial. This protective function has also indirect physiological and biochemical effects. We did not observe significant changes in many of the studied parameters, as chlorophylls *in vivo*, chlorophyll *a* fluorescence parameters, the secondary metabolites (anthocyanins and phenolic index), and lipid peroxidation. Previous studies on pepper plants (Jufri and Sulistyono, 2016) reported that the application of antitranspirants did not have significant effect on physiological processes (photosynthesis rate, stomata conductance, transpiration, leaf temperature), growth parameter and yield. This supports the hypothesis that the responses obtained in our study are the result of the indirect effects of the antitranspirant. In cold sensitive species such as cucumber, tomato, cotton, soybean, and beans, at low temperatures, stomata are locked open and plants are unable to normally respond to leaf water deficit, increasing chilling injury (Allen and Ort, 2001). In an experiment carried out on artichoke plants, the foliar application of film-forming antitranspirants was not as effective as ABA treatment in protecting plants from heat and drought stress, and this suggests that the antitranspirant effect could be species dependent and influenced by the leaf morphology (Shinohara and

Leskovar, 2014). Iriti et al. (2009) also observed that none of the antitranspirants tested on bean plants led to significant changes in most of the chlorophyll *a* fluorescence parameters. The authors concluded that the activity of the commercial natural antitranspirant, made with resins, used in their experiments, was based on the formation of a film over the leaf and did not depend on the reduction of stomatal opening. Similarly, Scudotherm® applied on the crops forms a semipermeable membrane that regulates water exchanges between the plant and the environment and vice versa. The antitranspirant avoided the anthocyanins and total phenols reduction and these results can be considered a positive effect of the treatment. A proteomic study demonstrated that chilled beans showed a down-regulation of phenylalanine ammonia lyase (PAL), the key enzyme of total phenols and anthocyanins (Badowiec and Weidner, 2014). We did not observe any variations in secondary metabolites. Beside secondary metabolism, also the primary metabolism is affected by low temperatures. Changes in the carbohydrate metabolism are often observable in case of cold stress in plants (Frankow-Lindberg, 2001; Beck et al., 2004; Liu et al., 2010; Watanabe and Ayugase, 2014; Yoon et al., 2017). In the same proteomic study reported above (Badowiec and Weidner, 2014), the highest up-regulated protein was the glyceraldehyde-3-phosphate dehydrogenase that is involved in the carbohydrate and energy metabolism. These data confirm the sugar variations observed in our experiment in control and stressed treatments. Scudotherm® influenced partially the sugar metabolism, allowing to maintain the sucrose concentration similar to unstressed control and lower than stressed plants. These results are the evidence that the antitranspirant protected the plants from the cold effects. In spinach, increased sucrose content after cold stress may be due to the up-regulation of the sucrose biosynthetic pathway which generally occurs during the freezing tolerance process (Strand et al., 2003; Yoon et al., 2017). Similarly, Hagen et al. (2009) observed increased levels of sucrose in curly kale subjected to cold stress. Johansen et al. (2016) reported that, after a short period with low (0-9 °C) growth temperature before harvest, it is possible to increase also the total sugar content, up to 10–15%, in swede roots. In our trial, during the first year, we recorded an increment of total sugars, in a more marked way, in stressed plants. Once again, Scudotherm® showed intermediate levels, suggesting that it is able to mitigate the negative consequences deriving from the cold exposure. Certainly, this is a preliminary study aimed at carrying out a first screening on the effects of Scudotherm® in case of low temperatures. In the future, it will be interesting to examine if and how Scudotherm® could affect growth-related parameters and yield, particularly influenced by this kind of abiotic stress. A more complete view on the efficacy and on the mode of action of this commercial product can also provide more targeted information to farmers.

5. Conclusions

The results of our study suggest that the protective role of Scudotherm® against cold is due to the reduction of transpiration that has a positive and indirect effect principally on the primary metabolism (sugars) of the bean plants. Under cold stress condition, compatible solutes, such as sugars, were accumulated in order to enhance the plants tolerance. Immediately after the stress, the Scudotherm® treatment showed intermediate levels of total sugars (year 2018) and sucrose (year 2019). These results indicate that the treatment protected the bean plants from the negative effects of cold. Moreover, the visual analysis of damages resulting from cold stress on leaves demonstrates the effectiveness of the Scudotherm® treatment.

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