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Improved Resilience and Metabolic Response of Transplanted Blackberry Plugs Using Chitosan Oligosaccharide Elicitor Treatment

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ABSTRACT

Transplanting of micro-propagated blackberry plugs in the field during late summer and early fall is a common nursery practice for commercial blackberry production. During this field transition, blackberry plants generally experience different stresses in addition to transplanting shock coupled with sudden exposure to low night temperature in the fall. Improving stress resilience of field transplanted blackberry plant by recruiting plant endogenous protective metabolic responses has significant merit. Therefore, the aim of this study was to improve stress resilience of newly transplanted “Chester Thornless” blackberry in the field during fall transition through stimulation of phenolic antioxidant and proline-linked metabolic responses by using bioprocessed chitosan oligosaccharide (COS) as an elicitor treatment. Fourteen weeks old blackberry plugs were transplanted in the field in late July and COS was sprayed weekly in the run-off for 6 weeks period after transplanting. Total soluble phenolic content, total antioxidant activity, total proline content, proline-dehydrogenase (PDH), and succinate dehydrogenase (SDH) enzyme activity of blackberry shoots were evaluated weekly during and one week after last COS application. Improvement in total soluble phenolic content and antioxidant activity based on ABTS free radical scavenging assay was observed in field transplanted blackberry at 6 and 7 weeks with COS elicitation treatment.

Key words: antioxidant; elicitor; phenolic; proline; transplanting shock

INTRODUCTION

Blackberry (*Rubus Spp.*) is an important edible berry crop from family *Rosaceae*. Due to its growing popularity among consumers commercial blackberry production is increasing rapidly in the United States and around the world. Overall, the current market value of the blackberry production in the United States is more than \$50 million, while the value of imported fresh blackberries is around \$207 million (AMRC 2015; NASS 2015). North America (USA, Mexico, and Canada) is the leading producer of blackberry in the world and its production is expected to increase significantly in the future to satisfy the growing consumer demand of blackberry either as a fresh berry or as a processed and canned product (Kaume et al. 2012). The sustainability and improvement of blackberry production in the future will largely depend on the development of new stress resilient cultivars along with improvement in production practices and post-harvest preservation strategies. Commercial blackberry cultivars are mostly classified according to their growth habits as erect, semi-erect, and trailing types and also as thorny and thornless cultivars (Clark and Finn 2011). As a temperate fruit crop, blackberry is generally tolerant to cold temperature, however semi-erect thornless cultivars are mostly cold sensitive. Among thornless semi-erect blackberry cultivars, “Chester Thornless” is the most stress-resilient cultivar with significant cold tolerance, and also resistant to cane blight (Weber 2013). However, at critical growth stages and during seasonal transitions (especially from late summer to autumn), blackberry cultivars including “Chester Thornless” are exposed to different abiotic and biotic stresses that are variable and unpredictable. Further, cultivation practices such as harrowing, transplanting, and pruning can mechanically damage root or shoot tissues and subsequent wound exposure can make plant more susceptible to other external stresses (Close et al. 2005).

Developing field-ready blackberry plugs in the greenhouse from micro-propagation is a common nursery practice, and this strategy is now being widely used for commercial and home-grown blackberry production (Najaf-Abadi and Hamidoghli 2009). After growing in the greenhouse, blackberry plugs are commonly transplanted in the field during late summer and early fall. During this transplanting process, blackberry plants generally experience some degree of transplanting shock (Demchak 2009). At this re-establishment phase, transplanting shock coupled with other environmental factors such as seasonal transition (late summer) potentially affect the growth, survival, and fitness of blackberry crop in the field. Therefore, improving resilience of blackberry during this transplanting phase by recruiting plants endogenous protective defense responses is important, especially to ensure better establishment, recovery, and subsequent improvement in vigor, fitness, and productivity of blackberry in the following fruiting year.

In general, plants adaptive response against environmental and other external stresses including transplanting shock coupled with seasonal transition potentially involves several physiological, metabolic, and structural adjustments (Kozłowski and Pallardy 2002). Biosynthesis of secondary metabolites, such as phenolic bioactives in plants under stress is part of an important metabolic response and also associated with other critical metabolic regulations including partition and allocation of carbon between different catabolic and anabolic needs and redox-linked cellular energy balance (Shetty 1997; Shetty and Wahlqvist 2004). Such redox-linked metabolic regulation involving protective function of phenolics is important to counter higher concentrations of reactive oxygen species (ROS) in the cells and to mitigate stress-induced oxidative breakdowns, which has significant relevance for improving establishment and recovery of plants after transplanting shock in the field (Choudhury et al. 2013). Phenolic

metabolites with high antioxidant potentials can neutralize ROS or can induce endogenous antioxidant enzyme response and thus can help plants to withstand stress-induced oxidative pressure associated with transplanting shock (Rice-Evans et al. 1997; Shetty 1997; Shalaby and Horwitz 2014) Further, protective function of phenolics and antioxidant enzymes is also associated with other metabolic responses of plants, such as higher accumulation of proline in the cytosol and its potential role as an active metabolic regulator in the mitochondria to support energy (ATP) synthesis under stress (Hare et al. 1999; Shetty and Wahlqvist 2004; Ben Rejeb et al. 2014).

Proline is the most abundant amino acid in plant cells and significantly accumulates far beyond cellular protein needs under abiotic and other external stresses (Hare et al. 1999). The protective function of proline under such stresses was earlier considered as an osmolyte to maintain solute balance in the cytosol and to protect cellular membrane (Kavi Kishor and Sreenivasulu 2014). However, the potential role of proline as an active metabolic regulator under external stresses in plants was not investigated and not explained until recently (Hare et al. 1999; Shetty and Wahlqvist 2004). In the cytosol, proline is synthesized from glutamate by a series of reduction reactions (Rayapati and Stewart 1991). During respiration process, oxidation reactions produce hydride ions, which help reduction of pyrroline-5-carboxylate (P5C) to proline in the cytosol (Rayapati and Stewart 1991; Hare et al. 1999; Shetty and Wahlqvist 2004). The accumulated proline in the cytosol could then enter into the mitochondria and can act as a reducing equivalent by donating electron instead of NADH to support oxidative phosphorylation for energy (ATP) synthesis (Hare et al. 1999; Shetty and Wahlqvist 2004). This energy generating metabolic role of proline potentially helps plants to generate ATP by supporting oxidative phosphorylation without driving energy expensive TCA/Krebs cycle and NADH

dependent respiration under induced stress conditions such as transplanting shock coupled with seasonal transition (Hare et al. 1999; Shetty and Wahlqvist 2004). Several redox-linked metabolic strategies have been developed to improve resilience of plants against external stresses by recruiting protective metabolic functions of proline and phenolic antioxidants (Shetty and Wahlqvist 2004; Sarkar and Shetty 2014). One such innovative metabolic strategy is the use of natural elicitors as seed, foliar, or root zone treatments to upregulate endogenous protective defense related pathways in plants to counter external stresses including transplanting shock.

Chitosan oligosaccharide (COS) as a natural bioprocessed elicitor has shown diverse functions including improvement of biotic and abiotic stress resilience in plant (Agrawal et al. 2002; Fan et al. 2010; Sarkar et al. 2010). Being a compound made from fungal cell walls or equivalent from marine sources, COS can trigger critical host adaptive protective responses in plants at very low doses without causing harmful effects (Kim and Rajapakse 2005; El Hadrami et al. 2010). Bioprocessed soluble COS as seed or foliar treatments have shown effective protection against different fungal pathogens, microbes, cold stress and can potentially stimulate secondary metabolite synthesis in plants for improving both biotic and abiotic stress tolerances (Khan et al. 2003; Prapagdee et al. 2007; Sarkar et al. 2010). Therefore use of COS as a natural elicitor to improve resilience of blackberry plugs in the field during transplanting and seasonal transition has significant merit. Based on this scientific rationale, the major aim of this study was to evaluate the impact and efficacy of COS as an elicitor treatment to improve resilience of newly transplanted “Chester Thornless” blackberry plugs during seasonal transition from late summer through early autumn after field transplanting. Further phenolic antioxidant and proline-linked metabolic responses were evaluated to determine the COS induced improvement in critical endogenous defense responses in newly transplanted blackberry plugs.

MATERIALS AND METHODS

Plant Material and Chitosan Oligosaccharide (COS) Treatments

Micro-propagated blackberry plants, var. “Chester Thornless” (*Rubus fruticosus* ‘Chester’), were planted into plug trays (cell size 1.5” x 2.75”) filled with a soilless peat-based potting mix in the greenhouse (in Whatley, MA-01093, USA) on April 23, 2007. The size of the blackberry plug was 3.8 cm × 7 cm with top growth range between 7.5 to 10 cm. A modified method from Broome and Zimmerman(1978) was used for the micro-propagation of blackberry. For greenhouse study, 14 weeks after planting, blackberry plants (var. “Chester Thornless”) were sprayed (mostly adaxial layer was exposed to spary) weekly with soluble bioprocessed COS (4 g/ L of water) from August 4th to September 16th. Chitosan oligosaccharide (COS-C) with 400~2,000 molecular weight and 80 mesh size (95%) (derived from shells of marine crustaceans) cross linked with ascorbic acid, obtained from Kong Poong Bio (Jeju, South Korea). For field experiment 14 weeks old blackberry plugs (var. “Chester Thornless”) were transplanted out to a nursery field on July 27th and sprayed weekly with soluble bioprocessed COS (4 g/ L of water), from August 4th to September 16th. The plants were sprayed to run off using a hand-held pressure sprayer (Solo 450 series hand held sprayer) with 15 psi approximate pressure. Control plants were sprayed with water. The average mean temperature was 20°C and total rainfall was 6.96 cm during the time of the experiment. However, field plants were also irrigated as needed. The experiment was carried out both in the greenhouse and in the field to evaluate the impact of transplanting in the field by comparing with the greenhouse grown plugs. Blackberry shoot sample were collected from both greenhouse and field experiment just before and one week after each COS application (from July 31st to September 23rd). Blackberry leaf sample was collected just one day before the COS spray application. Biological replicates representing individual

plants were used in triplicates and leaf samples were collected randomly from the individual plants of the respective treatment block. Newest fully expanded leaf just below shoot tip was collected and immediately extracted following grinding on the same day (within 3 hours). From the randomly mixed leaf sample 200 mg and 50 mg of leaf sample were separated for the extraction.

Total Phenolics Assay

The total soluble phenolic content of blackberry shoot samples were determined by an assay modified from Shetty et al. (1995). A quantity of 50 mg (fresh weight-FW) blackberry leaf tissue was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48-h. After 48-h, the sample was homogenized and centrifuged at 12,225 g for 10 min. Then 0.5 mL of sample supernatant was diluted with 0.5 mL of distilled water and transferred into a test tube with 1 mL of 95% ethanol and 5 mL of distilled water. Then 0.5 mL of 50% (v/v) Folin-Ciocalteu reagent (Sigma-Aldrich, St Louis, MO, USA) was added to the test tube, mixed and left to sit for 5 mins. After that 1 mL of 5% Na_2CO_3 was added and mixed and incubate for 60 mins in the dark. Absorbance was read at 725nm. Standard curves were created using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total soluble phenolic content and expressed as μg equivalents of gallic acid per gram fresh weight (FW) of shoot sample.

Antioxidant Activity by 2,2-diphenyl-1-picrylhydrazyl Radical (DPPH) Inhibition Assay

Same extraction from the total soluble phenolic content assay was used to determine antioxidant activity by DPPH free radical scavenging assay (Kwon et al. 2009). To 1.25 mL of 60 μM DPPH (2,2- Diphenyl-1-picrylhydrazyl, 1898-66-4, Sigma-Aldrich, St. Louis, MO, USA)

in ethanol, 0.250 mL of shoot culture extract in 95% ethanol was added, the decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The readings were compared with the controls, which contained 0.250 mL of 95 % ethanol instead of the extract.

The % inhibition was calculated by:

$$\% \text{ inhibition} = \left(\left[\frac{A_{517}^{\text{Control}} - A_{517}^{\text{Extract}}}{A_{517}^{\text{Control}}} \right] \right) \times 100$$

Antioxidant Activity by 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Inhibition Assay

Total antioxidant activity was also measured using the ABTS radical cation decolorization assay and with 10 times dilution of blackberry leaf sample (Re et al. 1999). ABTS radical cation was prepared by mixing 5 mL of 7 mM ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 30931-67-0, Sigma-Aldrich, St. Louis, MO, USA) solution with 88 μ L of 140 mM $K_2S_2O_8$ solution. This mixture is then stored in the dark at room temperature for 12 – 16 hours before using. Prior to the assay, this mixture is diluted with ethanol at an approximate ratio of 1:88 (ABTS : ethanol) and adjusted to yield an absorbance at 734nm of 0.70 ± 0.02 . A volume of 1 mL ABTS was added to 50 μ L of extract and the mixture was vortexed for 30s. The sample was incubated at room temperature for 2.5 minutes and then the absorbance was measured at 734 nm. The antioxidant activity of the extracts was expressed as % inhibition of ABTS radical formation and was calculated using the following formula:

$$\% \text{ inhibition} = \left(\left[\frac{A_{517}^{\text{Control}} - A_{517}^{\text{Extract}}}{A_{517}^{\text{Control}}} \right] \right) \times 100$$

Enzyme Extraction

A cold pestle and motor was used to thoroughly grind 100 mg of the blackberry leaf tissue in 2 mL of cold enzyme extraction buffer [0.5 % polyvinylpyrrolidone (PVP), 3 mM EDTA, 0.1 M potassium phosphate buffer (prepared by mixing monobasic- KH_2PO_4 to dibasic- K_2HPO_4) with pH 7.5]. The sample was then centrifuged at 12000 x g for 15 min at 2-5 °C and stored on ice. The supernatant was used for further biochemical analysis.

Total Protein Assay

Protein content was measured by the method of Bradford assay (Bradford 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 100 μL of the leaf tissue extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 μL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

Analysis of Proline

The proline content of the blackberry leaf tissue was determined using high performance liquid chromatography (HPLC) method (Sarkar et al. 2009). The HPLC analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a packing material of 5 μm particle size and at temperature 24°C. The leaf tissue extracts were eluted out in an isocratic manner with a mobile phase consisting of 20 mM phosphoric acid (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min⁻¹ and detected at 210 nm. L-Proline (Sigma

chemicals, St. Louis, MO) dissolved in the 20 mM potassium phosphate solution was used to calibrate the standard curve (Kwon et al. 2009). The amount of proline in the sample was reported as mg of proline per milliliter and converted to $\mu\text{g mg}^{-1}$ FW.

Proline Dehydrogenase (PDH) Assay

A modified method described by Costilow and Cooper (1978) was used to assay the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD (β -nicotinamide adenine dinucleotide hydrate, 53-84-9, Sigma-Aldrich, St. Louis, MO, USA) was used. To 1 mL of this reaction mixture, 200 μL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 °C. The absorbance was recorded at zero time and then after 3 min. In this spectrophotometric assay, one unit of enzyme activity is equal to the amount causing an increase in absorbance of 0.01 per min at 340 nm (1.0 cm light path).

Succinate Dehydrogenase (SDH) Assay

Modified method described by (Bregman 1987) was used to assay the activity of succinate dehydrogenase. The tissue extract suspension was diluted with 2.0 mL of enzyme extraction buffer. The enzyme sample was then assayed at room temperature for succinate dehydrogenase activity. The assay mixture consisted of the following: 1.0 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 μL of 0.15 M sodium succinate (pH 7.0); 40 μL of 0.2 M sodium azide; and 10 mL of 6.0 mg/mL DCPIP (Dichlorophenolindophenol). This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 μL of the enzyme sample was added. The rate of change in

absorbance per minute was used to quantify the enzyme in the mixture using the extinction coefficient of DCPIP ($19.1 \text{ mM}^{-1}\text{cm}^{-1}$).

Statistical Analysis

All biochemical analysis was performed in triplicates. Means, standard errors and standard deviations were calculated using Microsoft Excel 2010. Analysis of variance (ANOVA) for the data was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). Based on the initial ANOVA results, significant differences in phenolics, proline, antioxidant, and enzyme (PDH and SDH) activities between elicitor treatments (water and COS) and between field and greenhouse grown blackberry plugs were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$) separately for all time points (week 1 through week 7).

RESULTS AND DISCUSSION

Total Soluble Phenolic Content

Exposure to environmental stress or wounding of tissues potentially results in an increase in oxidative stress, which subsequently leads to redox-linked metabolic breakdown in plant cells (Walter et al. 2013). Therefore, restoring redox homeostasis is essential for post-stress recovery and to determine overall fitness and resilience of plants against biotic and abiotic stresses (Kapoor et al. 2015). Such redox-linked metabolic response associated with the stress-recovery process potentially involves stimulation of phenolic antioxidants and upregulation of other critical protective defense related anabolic pathways such as gateway pentose phosphate pathway

(PPP) (Shetty and Wahlqvist 2004). Therefore, soluble phenolic content of plant tissues is an effective bio-marker to understand the overall redox-linked metabolic responses of plant under external stresses such as transplanting shock coupled with seasonal transition. Based on this scientific rationale, total soluble phenolic content of leaf and shoot tissues from newly transplanted blackberry plugs was evaluated before and after spraying with bioprocessed natural elicitor (COS). Shoot samples of blackberry plugs both from the field and the greenhouse were evaluated and compared to determine the potential impact of COS elicitor treatment for improving stress resilience after transplanting in the field.

Overall, both in the greenhouse and in the field, total soluble phenolic content of blackberry shoots increased steadily with spraying and highest total soluble phenolic content was observed after 3 weeks of spraying (irrespective of the spraying treatments-control and COS) (Figure 1A & 1B). Further, from 3 weeks to 6 weeks after transplanting, the average baseline value of total soluble phenolic content of the field grown blackberry was slightly higher when compared with the greenhouse grown blackberry plugs (not-transplanted), however the difference was not statistically significant. Transplanting shock along with changes in macro- and micro-climate in the field potentially resulted in slightly higher baseline value of total soluble phenolic content in transplanted blackberry. Such metabolic response involving stimulation of biosynthesis of protective secondary metabolites might have relevance for improving resilience and fitness of blackberry plants after transplanting. Not only just increased concentration of phenolics but also the subsequent polymerization of phenolics potentially have significant relevance for structural adjustments, which is essential for improving stress resilience during recovery and re-establishment phase after transplanting (Randhir et al. 2002; Shetty and

Wahlqvist 2004). Alteration in secondary metabolism and increased concentration of related terpenoids after transplanting was previously reported in pine seedlings (Sallas et al. 1999).

In this study, under greenhouse condition higher total soluble phenolic content of blackberry shoots was observed with COS elicitor treatment after 4 and 5 weeks ($p < 0.05$) of spraying, while after 6 weeks no significant differences in phenolic content between treatments (control & COS) was observed. However in the field, COS elicitor treatment resulted in significantly higher ($p < 0.05$) total soluble phenolic content in transplanted blackberry plugs at 4, 6, and 7 weeks. Higher phenolic content in transplanted blackberry after COS spraying has potential relevance for using COS as a natural elicitor to stimulate phenolic biosynthesis and to subsequently improve resilience against abiotic and biotic stresses after transplanting. Improved resilience against abiotic and biotic stresses through up-regulation of phenolic biosynthesis was observed previously with COS application (Khan et al. 2003; Prapagdee et al. 2007; Sarkar et al. 2010). Therefore, application of COS as a run-off or foliar treatment to improve resilience of blackberry after transplanting especially during the seasonal transition has significant merit. However, this novel elicitation strategy with COS has to be further evaluated with other blackberry cultivars and in different hardiness zones of the United States in the future.

Total Antioxidant Activity

Stress and wounding response of plant in part also involves other enzymatic and non-enzymatic antioxidant mobilization including phenolics, and such antioxidant-linked metabolic response eventually helps plant to mitigate stress-induced breakdown of redox-homeostasis (Foyer and Noctor 2005). Therefore, antioxidant activity is an important indicator to understand overall stress response of plant and its potential resilience against external stresses such as

transplanting shock coupled with seasonal transition. Total antioxidant activity of blackberry plugs with and without COS elicitor treatments after transplanting were evaluated using two different free radical scavenging assays (2,2-diphenyl-1-picrylhydrazyl-DPPH & 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)- ABTS). Overall, very high antioxidant activity (70-90% inhibition) was observed in blackberry shoots both from the greenhouse and from the field. No significant differences in total antioxidant activity based upon DPPH free radical scavenging assay was observed between spraying treatments and between growing conditions (transplanted in field and greenhouse) (Figure 2A & 2B). Further the antioxidant activity (DPPH based) between blackberry plugs from field and greenhouse was compared after adjusting to 50µg/mL phenolic content. The rationale for adjusting to specific phenolic content was based on the hypothesis that phenolic metabolites predominantly contribute to the antioxidant activity of blackberry. Following this rationale the sample was diluted and adjusted to a quantifiable range in order to establish the relationship between total phenolic content and associated antioxidant activity of blackberry. Even when phenolic content was adjusted to 50µg/mL, no significant differences in total antioxidant activity (from week 2 through week 7) between treatments were observed with DPPH free radical scavenging assay (Figure 3A & 3B). High baseline value of DPPH % inhibition might have contributed for not observing any significant further increases and differences between spraying treatments or with transplanting shock in "Chester Thornless" blackberry.

However, significant differences ($p < 0.05$) in total antioxidant activity of blackberry shoots among spraying treatments and growing conditions were observed with ABTS free radical scavenging assay (Figure 4A & 4B) (from week 2 through week 7). Total antioxidant activity based on the total phenolic content and measured by the ABTS free radical scavenging assay was

highest at week 4 (after 3 weeks of spraying) for both control and COS treated blackberry plugs. Such high total antioxidant activity also coincided with an increase in total phenolic content of blackberry shoots with both spraying treatments. Based on ABTS free radical scavenging assay, significantly higher ($p < 0.05$) total antioxidant activity was also observed in field transplanted blackberry when compared with the greenhouse grown blackberry plugs (based on total phenolic content and when phenolic adjusted to 20 $\mu\text{g/mL}$) (Figure 4A, 4B, 5A, & 5B). Phenolic content of the sample was adjusted to 20 $\mu\text{g/mL}$ to determine the contribution of phenolic towards antioxidant activity of blackberry and to measure in a quantifiable range. In field transplanted blackberry, COS elicitor treatment resulted in higher ($p < 0.05$) total antioxidant activity (ABTS % inhibition) at 5, 6, and 7 weeks when compared to the control. There was a decline in antioxidant activity for control plants from week 5 through week 6 whereas, COS treated blackberry exhibited a sustained antioxidant response from week 4 through week 7. Higher antioxidant activity and improved cold tolerance in creeping bentgrass (*Agrostis spp.*) after COS foliar application was also observed previously (Sarkar et al. 2010). Improvement in total antioxidant activity also positively correlated with total soluble phenolic content after COS spraying, and might have significant relevance for improving stress resilience in blackberry after field transplanting and especially during seasonal transition of fall, when day and night temperature vary significantly. The differences between DPPH and ABTS based assays is potentially linked to type of phenolics associated with radical scavenging and where water soluble phenolics are more responsive in ABTS assay (Re et al. 1999).

In general, blackberry plants continue to grow during fall (September and October), as long as temperatures do not drop below freezing. However, as canes elongate, begin to trail, and reach the soil, the shoot tips will stop growing and begin to root. The onset of root initiation

could trigger the increase in phenolic content and antioxidant activity to counter higher levels of rooting and transplanting shock-induced oxidative stress. Weekly applications of COS to field grown “Chester Thornless” blackberry plants could have stimulated the stress response to increase phenolic biosynthesis and associated antioxidant activity to counter such oxidative pressure. On the contrary, in the greenhouse, blackberry plugs continue to grow until the temperatures are lowered to slow and eventually cease shoot growth. The shoot tips generally do not root in the greenhouse and therefore do not need to stimulate stress related metabolic responses including phenolic-linked antioxidant response. Therefore increased phenolic content along with increased antioxidant activity in field transplanted blackberry after COS treatment would have more relevance for improving resilience of blackberry during seasonal transition of fall. Such metabolic response involving phenolic and associated antioxidant activity can potentially help blackberry to recover from overall stress and to improve vigor and fitness after transplanting shock in the field.

Total Proline Content and Proline Dehydrogenase (PDH) Activity

In general, under biotic and abiotic stresses stimulation of proline synthesis in the cytosol is a common stress-induced metabolic response of plant (Fabro et al. 2004; Claussen 2005). Such higher accumulation of proline either as an osmolyte in the cytosol or its potential role as a reducing equivalent instead of the NADH in the mitochondria for energy synthesis (ATP) in part is plant endogenous defense responses to counter stress-induced metabolic breakdowns and to protect against subsequent damages of cellular organelles (Hare et al. 1999; Shetty and Wahlqvist 2004). Further, synthesis of proline in the cytosol is also associated with the gateway pentose phosphate pathway (PPP) which potentially drive carbon flux towards phenolic

biosynthesis via shikimate and phenylpropanoid pathways (Shetty and Wahlqvist 2004).

Therefore, phenolic-linked antioxidant enzyme response of plant under stress is also potentially associated with the synthesis of proline (Shetty and Wahlqvist 2004). Based on this scientific rationale, we hypothesized that COS elicitor treatment would stimulate proline synthesis in the cytosol which would drive carbon flux towards phenolic biosynthesis through upregulation of gateway PPP in field transplanted blackberry (Shetty and Wahlqvist 2004). Further, subsequent increase in oxidation of proline in the mitochondria with the help of proline dehydrogenase (PDH) would eventually help to conserve more energy by replacing NADH to support oxidative phosphorylation of respiration, which is an energy expensive process and not an ideal metabolic response during stress-recovery (Hare et al. 1999; Shetty and Wahlqvist 2004).

In this study, field grown “Chester Thornless” blackberry had higher levels of proline throughout the 8 week period irrespective of the spraying treatments (Figure 6A). Even in the greenhouse proline content of blackberry plugs remained steady from week 1 (August 1) through week 7 (September 23) (Figure 6B). Just after transplanting slightly higher proline content was observed in field grown blackberry when compared with the greenhouse grown blackberry plugs. This result potentially indicates a slight stimulation of proline synthesis in blackberry to counter transplanting shock in the field. However, COS spraying treatment did not result in any further stimulation of proline in the blackberry shoots when compared with the control. Higher baseline proline content may have contributed for not finding any significant differences between treatments both in the greenhouse and in the field. No proline was detected in blackberry plants from the greenhouse with COS application at week 4. Although increased accumulation of proline may indicate a positive response to a stressful condition in plant, maintaining a constant level of proline may also be indicative of plants adaptation to stress. Proline metabolism at a

sustained high level with its link to gateway PPP will drive alternative energy production and biosynthesis of secondary metabolites, which is potentially essential for the maintenance of cellular redox balance after transplanting shock especially during seasonal transition.

While proline content remained steady, the PDH activity of blackberry varied significantly during 8 weeks period (Figure 7A & 7B). In the field transplanted blackberry the highest PDH activity was observed at week 5 (September 8) for both control and COS treated plants. Proline content did not correlate with the PDH activity for COS treated blackberry plants. However in the greenhouse PDH activity was highest at week 5 (September 8) for control plants and at week 4 (September 1) for COS treated plants ($p < 0.05$). Significant variations and higher PDH activity at week 4 and 5 potentially indicate oxidation of proline in the mitochondria. However changes in PDH activity did not correlate with the proline content of the blackberry plugs. The fluctuations of PDH activity might be due to the variations in the environmental conditions (especially night temperature) and response of the blackberry plant to such changing environment. Additionally, the oxidation of proline in the mitochondria in specific stages and its relevant metabolic flux during transition and recovery of blackberry plant might have resulted in such wide variations in PDH activity. Therefore proline-linked metabolic response of field transplanted “Chester Thornless” blackberry after COS spraying is not conclusive in this study and needs further evaluation with other blackberry cultivars and under different stress conditions.

Succinate Dehydrogenase (SDH) Activity

Succinate dehydrogenase is an important enzyme of the Tricarboxylic Acid Cycle-TCA/Kreb's cycle and activity of SDH enzyme can indicate the rate of energy expending

catabolic respiration process and active growth in plants (Cooley and Vermaas 2001). Overall, similar to the total soluble phenolic content, SDH activity of blackberry shoots both from the greenhouse and the field increased steadily from week 1 to week 4 after initiation of the spraying treatment (Figure 8A & 8B). This result suggests a potential increase in respiration rate and active growth in “Chester Thornless” blackberry during this seasonal transition and with spraying. There is a usual reduction in SDH activity with decreasing temperatures and cold acclimated plants generally exhibit lower SDH activity than actively growing plants (Atkin and Tjoelker 2003). In field transplanted “Chester Thornless” blackberry, COS elicitor treatment resulted in significantly higher SDH activity ($p < 0.05$) when compared to the control at week 7, suggesting a potential stimulation of the TCA cycle and respiration rate. However, unlike the COS treated blackberry, SDH activity of the control plants began to decline at week 7 which indicated a reduction in active growth with lowering night temperature. Higher SDH activity in field transplanted blackberry after COS elicitor treatment suggest a continuation of active growth and potential resistance against rooting and winter dormancy. Similarly, in the greenhouse grown blackberry plugs, SDH activity remained steady at week 4 through week 8 suggesting a continuation of active growth with higher respiration rate. Higher rate of respiration of blackberry plugs during this seasonal transition especially with COS elicitation treatment might be due to the increasing demand for energy (ATP) synthesis through oxidative phosphorylation, which has significant relevance for different metabolic adjustments to support active growth during seasonal transition of fall.

CONCLUSION

Blackberry is a widely cultivated small fruit crop which continues to grow during the shortening days of autumn. Transplanting of micro-propagated blackberry plugs in the field during late summer and early fall is a common cultivation practice which potentially exposes blackberry plant to several external stresses including transplanting shock coupled with rapid variations in day and night temperature. Therefore improving resilience of field transplanted blackberry during seasonal transition is essential for recovery and better establishment of blackberry plants prior to the winter. Though this is a one year study, a novel elicitation strategy with soluble bioprocessed COS was used to improve resilience of field transplanted blackberry through stimulation of phenolic antioxidant-linked metabolic response, which is a part of stress-induced adaptive responses of plant. This metabolic rationale has merit for further deeper study. Higher phenolic content and total antioxidant activity based on ABTS free radical scavenging assay was observed in field transplanted blackberry with COS elicitation treatment at 6 and 7 weeks after transplanting. Similar to the soluble phenolic content, higher SDH activity was also observed with COS elicitation treatment at week 7. The proline content and PDH activity of blackberry after transplanting varied significantly and its potential role to induce stress resilience was not conclusive in this study. Overall results suggested that COS elicitation strategy might have relevance to improve resilience of field transplanted blackberry through stimulation of phenolic biosynthesis and mitochondrial energy (ATP) synthesis by up-regulation of TCA cycle driven respiration process. However, other primocane and florican blackberry cultivars with COS elicitation treatments must be evaluated in the future to further prove this metabolically-driven concept, especially under different abiotic and biotic stresses and with multi-year study. Further, metabolic responses involving gateway pentose phosphate, and phenylpropanoid

pathway and activity of individual antioxidant enzymes such as superoxide dismutase, catalase, guaiacol peroxidase, glutathione synthase, NADPH oxidase should also be evaluated for better understanding of adaptive response of blackberry cultivars under stress including transplanting shock coupled with seasonal transition.

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Figure Captions

Figure 1. Total soluble phenolic content ($\mu\text{g}/\text{mg}$ fresh weight) of blackberry shoots from the field (1A) and from the greenhouse (1B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in phenolic content between elicitor treatments for each time point at $p < 0.05$. Vertical bars represent standard error (SE).

Figure 2. Total antioxidant activity (DPPH % inhibition based on total phenolic content) of blackberry shoots from the field (2A) and from the greenhouse (2B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in DPPH based antioxidant activity between treatments. Vertical bars represent standard error (SE).

Figure 3. Total antioxidant activity (DPPH % inhibition based on the phenolic content adjusted to $50 \mu\text{g}/\text{mL}$) of blackberry shoots from the field (3A) and from the greenhouse (3B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in DPPH based antioxidant activity between treatments. Vertical bars represent standard error (SE).

Figure 4. Total antioxidant activity (ABTS % inhibition based on total phenolic content) of blackberry shoots from the field (4A) and from the greenhouse (4B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in ABTS based antioxidant activity between elicitor treatments for each time point at $p < 0.05$. Vertical bars represent standard error (SE).

Figure 5. Total antioxidant activity (ABTS % inhibition based on the phenolic content adjusted to 20 µg/mL) of blackberry shoots from the field (5A) and from the greenhouse (5B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in ABTS based antioxidant activity between elicitor treatments for each time point at $p < 0.05$. Vertical bars represent standard error (SE).

Figure 6. Total proline content (µg/mg fresh weight) of blackberry shoots from the field (6A) and from the greenhouse (6B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). The proline data of the COS treated plant from the greenhouse (6B) is missing. No significant differences were observed in proline content between treatments. Vertical bars represent standard error (SE).

Figure 7. Proline dehydrogenase (PDH) activity (Units/mg protein) of blackberry shoots from the field (7A) and from the greenhouse (7B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in PDH activity between elicitor treatments for each time point at $p < 0.05$. Vertical bars represent standard error (SE).

Figure 8. Succinate dehydrogenase (SDH) activity (nmol/mg protein) of blackberry shoots from the field (8A) and from the greenhouse (8B) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in SDH activity between elicitor treatments for each time point at $p < 0.05$. Vertical bars represent standard error (SE).

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