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Research Application Summary

Changes in respiration pathways during hydrogen cyanamide-induced terminal budbreak of Cripps' Pink

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Abstract

Apple trees that do not receive adequate chill during winter undergo improper release of endodormancy resulting in decreased bud break, and uneven and delayed blooming that impacts negatively on yield. To overcome this, chemical rest breaking agents are used to artificially release the buds from endodormancy and synchronise bud break. This study was conducted to investigate the effects of hydrogen cyanamide (HC) (Dormex™) and oil on the total respiration rate and the rate of four respiratory pathways (tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), alternative pathway (ALT) and cytochrome C (CYT) pathways). The HC-treated endodormant terminal buds of mature Cripp's Pink trees were randomly collected from commercial orchard in Elgin (warm winter area; ±700 CU). Control shoots were covered with plastic bags during the treatment. The respiration rate was determined by using a Clark-type oxygen electrode fitted to a liquid filled chamber containing the buds. The respiration rate of the different pathways was measured by using inhibitors targeting the specific pathways. Means were compared using a two-way ANOVA. The results showed that Dormex™ and oil treatment cause an increase in the respiration rate in the TCA and CYT pathways reaching a maximum at the budbreak. The untreated buds continued to show a crippled respiration rate with an increasing use of the ALT pathway. The study concludes that the treatment with Dormex™ and oil prevents delayed foliation and unsynchronised, poor bud break by increasing the respiration rate of bud tissue via the TCA and CYT pathways to provide energy for growth.

Key words: Apple buds, Dormex™, endodormancy, hydrogen cyanamide, respiration

Résumé

Les pommiers ne recevant pas une fraicheur adéquate en hiver, subissent une libération inappropriée de l'endo-dormance, ce qui conduit à une diminution des bourgeons et une floraison irrégulière et retardée qui a un impact négatif sur le rendement. Pour surmonter cela, des agents chimiques de levée de la dormance sont utilisés pour libérer artificiellement les bourgeons de et synchroniser leur ouverture. Cette étude a été menée pour évaluer les effets du cyanamide d'hydrogène (HC) (Dormex ™) et de l'huile sur le taux respiratoire total et le taux de quatre voies respiratoires (cycle acide tricarboxylique (TCA), voie des

pentoses phosphates (PPP), voie alternative (ALT) et la voie du cytochrome C (CYT)). Les bourgeons terminaux dormants des tiges matures de Cripp's Pink traités avec le HC ont été prélevés au hasard dans un verger commercial à Elgin (zone chaude d'hiver, ± 700 UT). Les pousses pour le témoin ont été recouvertes de sacs en plastique pendant le traitement. Le taux de respiration a été déterminé en utilisant une électrode à oxygène de type Clark ajustée à une chambre remplie de liquide contenant les bourgeons. Le taux de respiration des différentes voies a été mesuré en utilisant des inhibiteurs ciblant les voies spécifiques. Les moyennes ont été comparées en utilisant une ANOVA à deux facteurs. Les résultats ont montré que Dormex ™ et le traitement à l'huile entraînent une augmentation du taux de respiration dans les voies TCA et CYT, conduisant à un maximum d'ouverture de bourgeons. L'étude conclut que le traitement avec Dormex ™ et l'huile empêche un retard de floraison et une ouverture non synchronisée des bourgeons en augmentant la vitesse de respiration du tissu de bourgeon par les voies de TCA et de CYT pour fournir l'énergie pour la croissance.

Mots clés: Bourgeons de pomme, Dormex ™, endo-dormance, hydrogène de cyanamide, respiration

Background

Global climatic change has been reported to influence a decrease of winter chill unit accumulation and an increase of temperature worldwide, including South Africa (Luedeling *et al.*, 2011; Midgley and Lötze, 2011; Atkinson *et al*., 2013). As with other temperate and perennial woody plants, deciduous trees harmonise their annual growth development with periodic environmental changes. These tree fruits undergo annually unfavourable winter conditions, and receive different chill accumulation depending on local and global climate. A dormant winter phase allows cultivated perennial trees to adapt to seasonal changes and to avoid damage from environmental stresses. Rohde and Bhalerao (2007) define bud dormancy as an "inability of a meristem to resume growth under favourable conditions". This physiologically dormant state was classified by Lang (1987) and Lang *et al*. (1987) under three successive phases: paradormancy, endodormancy, and ecodormancy. Yamane (2014) described environmental factors that affect the seasonal dormancy depth and a correlation between genetic, biochemical and molecular biological networks that are synchronized and associated with bud dormancy progression. The author reported that chilling accumulation and dormancy release are the key factors that can influence a positive or negative effect on fruit yield.

In most temperate fruits, a specific amount of winter chill accumulation is known to induce dormancy release. In warm winter areas the chill requirement of some of these deciduous fruit crops is not met due to inadequate chill accumulation (Cook and Jacobs, 1999). The majority of published studies (Atkinson *et al*., 2013; Melke, 2015) on deciduous crops grown in warm areas report symptoms such as delayed foliation, unbranched shoots, low budbreak, delayed flowering, variable fruit maturity and abnormal fruits. Under South African conditions inadequate chill accumulation has been found in some apple production areas of the Western Cape (Cook and Jacobs, 2000; Labuschagne *et al*., 2000). Currently the use of a chemical rest breaking agent in areas with warm winter conditions is vital to partially compensate for

the lack of chill, break endodormancy and ensure acceptable levels of bud break needed to sustain profitable yields (Seif El-Yazal and Rady, 2012).

Hydrogen cyanamide (Dormex™) is the most useful chemical rest breaking treatment to release dormancy and is used in many crops (Seif El-Yazal and Rady, 2012). Dormex™ is found not only to increase and induce synchronised bud break (Halaly *et al*., 2008; Babita and Rana, 2013) but also to advance the date of bud break in kiwifruit (McPherson *et al*., 1999), for instance in grapevines (Nir *et al*., 1986) and pistachio trees (Ghrab and Ben Mimoun, 2014). Hydrogen cyanamide (HC) was found to increase proline content and biogenic amines (tyramine, tryptamine, histamine, methylbutylamine and serotonin) in floral dormant buds of apple trees (Mohamed *et al*., 2013). Seif El-Yazal and Rady (2012) found a significant increase in the nitrogen fraction and levels of polyamines in treated buds of 'Anna' apple trees during dormancy. The applications of HC activate oxidative stress and indirectly affect respiratory disturbances which are related to the release of endodormancy (Or *et al*., 2000; Perez *et al*., 2008). This oxidative stress is followed by increased levels of antioxidants, possibly to prevent damage to the plant when it commences growth (Wisniewski *et al*., 1996). Stress signals, induced by HC and associated with dormancy release, were sensed by the SNF-like protein kinase GDBRPK, detected in RNA extracted from purified mitochondria for a period of 8 to 14 days after treatment (Perez *et al*., 2008). Or *et al*. (2000) also confirmed that this transcript for an SNF-like protein kinase is up-regulated by HC application to dormant grape buds. In fact, Dormex™ decreases the activity of the enzyme catalase (Nir and Lavee, 1993). The authors showed that the inhibition of catalase with Dormex™ causes an increase in hydrogen peroxidase and induces H_2O_2 levels in plant which consequently maintains NADP in its oxidized form and eventually activates G-6PDH and 6-PGDH enzymes. Babita and Rana (2013) stated that via this sequence of reactions in pentose phosphate pathway (PPP), a range of substances (RNA, DNA, pentose sugars etc.) responsible for new growth in a plant are produced at higher rates. The fact that the rest breaking agents application induces stresses, it is needed to investigate total respiration rate and respiration pathway in apple dormant terminal bud treated with Hydrogen cyanamide and oil. In this study, changes in the respiration rate and respiration pathways (TCA, PPP, CYT, ALT) of apple buds dormancy were determined in order to monitor the effect of Dormex™ and oil respiration changes.

Study description

This study was conducted in 2015 (end of winter-beginning spring) on a commercial farm in the warm winter region (± 770 CU, Utah model) of Elgin (34ºS, 305m) in the Western Cape. Shoot from 'Cripps' Pink' trees were randomly selected in the orchard and removed at two day intervals after commercial treatment with 0.5% Dormex™ and 3% oil until bud break. Control shoots were covered with plastic bags during the treatment. Shoot were transported to the laboratory where respiration assays were performed within 24 hours after removal from the tree. Terminal buds were investigated using a Clark type oxygen electrode fitted to an Oxy-Graph instrument (Hansatech Instruments Ltd., England). Assay were conducted in the dark at 20ºC and repeated using 12 replicates. The total respiration rate of the bud tissue were determined for both the treated and control buds followed by the use of various

respiratory inhibitors to block individual pathways following the method used by Tan *et al.* (2013). Malonic acid (competitive inhibitor of succinate dehydrogenase in the TCA), Na_3PO_4 (inhibitor of glucose-6-phosphate dehydrogenase in the PPP), KCN (inhibitor of cytochrome oxidase in the CYT), and salicylhydroxamic acid (SHAM) (inhibitor of cytochrome alternative oxidase in the ALT) were used as respiratory inhibitors (Bi *et al*., 2011; Tan *et al*., 2013). From this the contribution of each pathway could be quantified relative to the total respiration according to the calculations used by Tan *et al.* (2013). Data were analysed by two-way analysis of variance (ANOVA) followed by Fisher's test in order to examine the significance of the observed differences using the XLSTAT package (XLSTAT Institute), and P values < 0.05 were considered as statistically significant.

Results

The results from the total respiration measurement (Fig. 1) indicates an interaction between the HC treatment and time (days) ($p<0.0001$). This implies that the changes brought about by the HC treatment is different from that of the control confirming that HC treatment increases the total respiration rate of the apple buds within 15 days after application. It is interesting to note that the control buds showed a significant increase in respiration after three days followed by a decrease resulting in the HC treated buds overtaking the respiration of the control at five days post treatment and has a significantly higher respiration at 9 nine days and maintains this higher level over the next four days whilst increasing more.

A similar pattern was present when observing the respiration of the TCA pathway, and the results indicates an interaction between the HC treatment and time (days) ($p<0.0001$) (Fig. 2). This means that changes in oxygen consumption in TCA pathway brought about by the HC treatment is different from that of the control confirming that HC treatment increases the total respiration rate of the apple buds within 15 days after application. Oxygen uptake in TCA pathway showed also a significant increase in respiration after three days followed by a decrease resulting in the HC treated buds overtaking the respiration of the control at

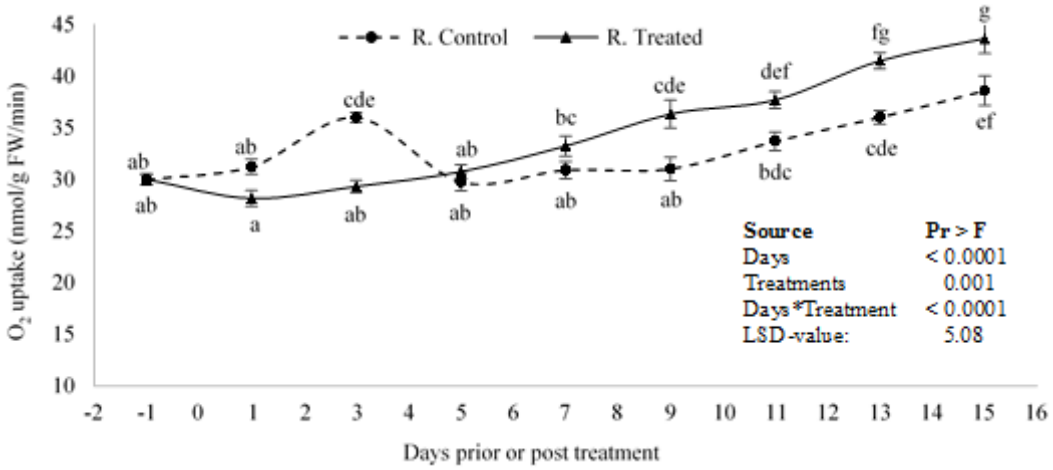

Figure 1. Effects of Dormex™ and oil on the total respiration of 'Cripps Pink' apple buds

five days post treatment and has a significantly higher respiration at 9 nine days and maintains this higher level over the next four days whilst increasing more.

The HC treatment did not alter the PPP pathway. Statistical interaction between the HC treatment and time (days) was not significant different from that of the control (Fig. 3). In both the control and the treated buds there was an increase in oxygen uptake in the PPP pathway over time, and were at their maximum during budbreak.

The CYT activity in the terminal buds showed much changes with a significant interaction between days and treatment (P<0.0001). In the control group, the oxygen uptake in CYT pathway slightly increased during the three first days, dropped till the 9th day about and then increased gradually (Fig. 4). However, HC-treated terminal dormant buds had reduced

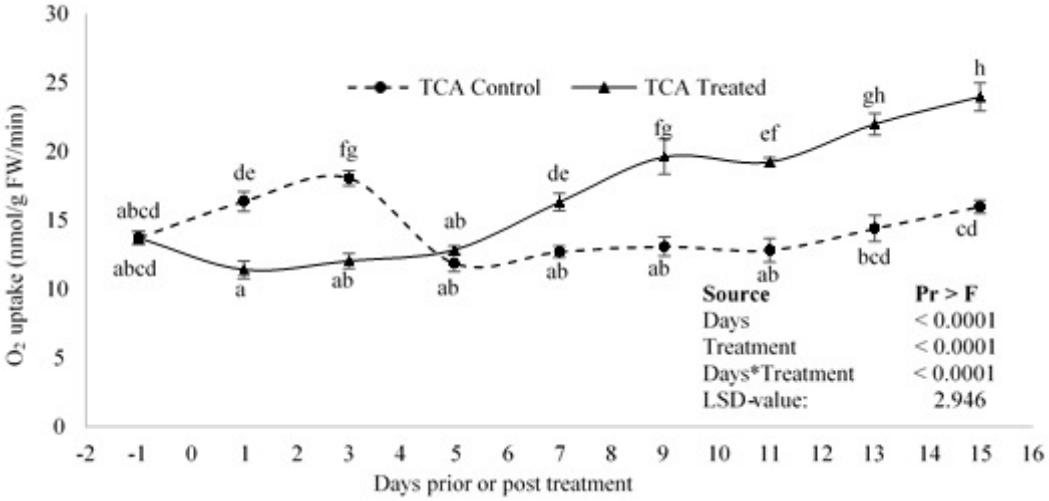

Figure 2. Effects of Dormex™ and oil on TCA of 'Cripps Pink' apple bud

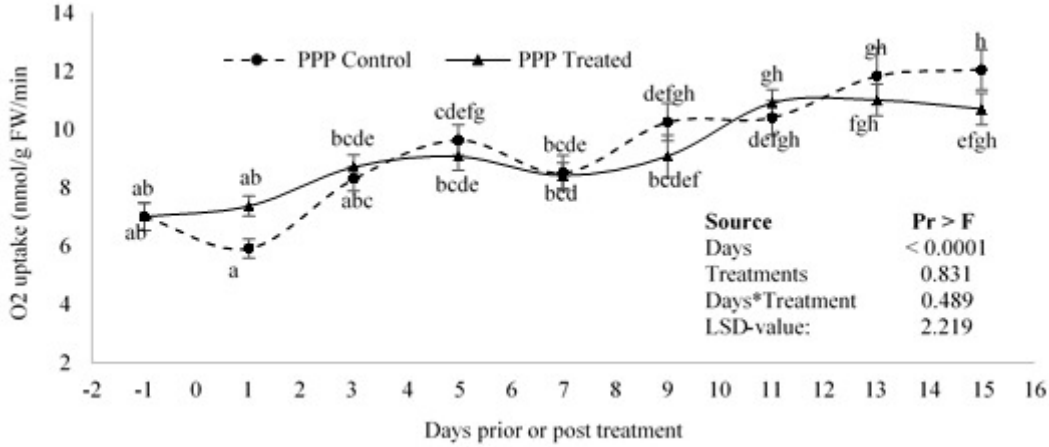

Figure 3. Effects of Dormex™ and oil on PPP of 'Cripps Pink' apple bud

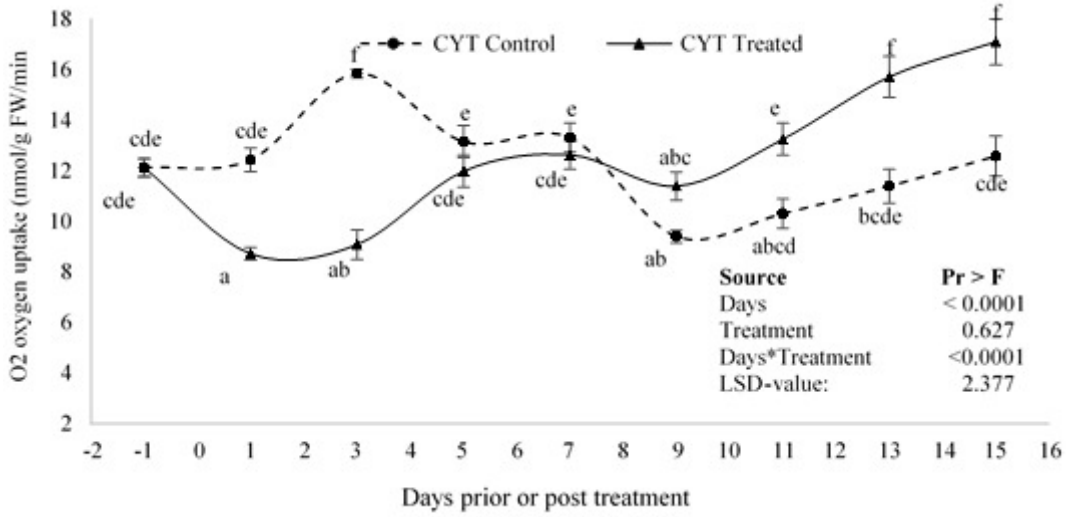

Figure 4. Effects of Dormex™ and oil on CYT of 'Cripps Pink' apple bud

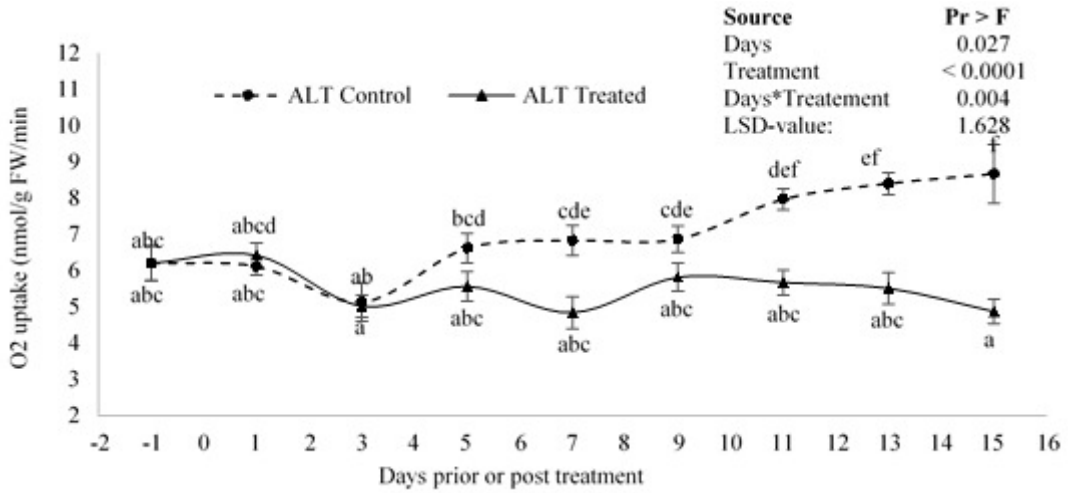

Figure 5. Effects of Dormex™ and oil on ALT of 'Cripps Pink' apple bud

respiration during the five first days which subsequently increased more compared to the control before budbreak (Fig. 4).

Results of oxygen uptake in ALT pathway indicate that there is a significant interaction between days and treatments (Fig. 5). This means that changes in oxygen consumption for HC treatment is different from that of the control. This indicates that HC treatment reduces oxygen uptake of the apple buds till budbreak, 15 days after HC application.

Discussion

Previous studies showed that this rest breaking agent activates oxidative stress and indirectly induces respiratory disturbances which are associated with the release of endodormancy (Or *et al*., 2000; Perez *et al*., 2008). Researchers reported that HC is considered to induce early bud break and increase bud break percentage (Theron *et al*., 2011). Erez (1987) suggested that HC and oil reduce the air exchange between the buds and the external environment by creation of low oxygen condition (or hypoxia) in the plant. Or *et al*. (2000) found that levels of the two enzymes for HC treated buds decreased during the first week after treatment. The current study agreed with the hypothesis. Significant changes in respiration were observed between HC treated buds and the untreated control. According to our results, total respiration is significantly affected with Dormex™ and oil. The inhibition of oxygen uptake observed during the first week (Figs. 1, 2 and 4) is probably related to the deficiency of oxygen (inhibition respiration) created by Dormex™ and oil. The anaerobic conditions induce a reversible inhibition of catalase activity that lead to oxidative potential (O^{2-} and H_2O_2 accumulation in dormant buds cells) and consequently disturb metabolic activities that might help buds to break dormancy (Nir and Lavee, 1993; Vergara *et al*., 2012). Normally, catalase controls the H_2O_2-concentration, and protects the redox systems from intoxication and irreparable damages. Wisniewski *et al*. (1996) proved that oxidative stress is followed by increased levels of antioxidants, possibly to prevent damage to the plant when it begins to grow. Significant increase in total respiration was observed after 5 days till budbreak. Young (1990) stated that apple buds exhibit an increase in respiration when induced to break endodormancy.

Dormex™ and oil had an effect on TCA (Fig. 2). This was supported by the differences observed between dormant buds treated and control. Panneerselvam *et al*. (2007) reported an increase in glycolysis and tricarboxylic acid (TCA) enzymes activities (Aldolase, Succinic dehydrogenase (SDH) and Malic dehydrogenase (MDH)) during germination in HC treated yam tubers and turmeric rhizomes. This increase in enzymes appeared even before the visible appearance of sprouting and their activities were at their maximum during sprouting. A short inhibition in TCA was observed during the first week after HC treatment compared to the control followed by a steady increase until budbreak It is known that Dormex™ inhibits catalase activity, which leads to an increase in H_2O_2 (Nir and Lavee, 1993). We suggest that this decrease in respiration could possibly be the effect of the accumulated H_2O_2 as Godon (1998) found a similar pattern whereby a repression of metabolic enzymes involved in glycolysis and the Krebs cycle was observed shortly after the exposure of yeast cells to H_2O_2.

It is suggested that the pentose phosphate pathway (PPP) plays an important regulatory role in the breaking of buds and seed dormancy. Bewley and Black (1978) indicated that the activity of this PPP pathway can be estimated by changes in the activity of Glucose-6-phosphate dehydrogenase (G6PDH), which regulates entry of glucose-6-P into the pathway. Previous studies demonstrated also that HC upregulates the expression of select genes encoding enzymes belonging to the ascorbate glutathione cycle, to the oxidative pentose phosphate pathway and a key enzyme (1,3-b-D-glucanase) for dormancy release (Perez *et*

al., 2009; Vergara and Pérez, 2010), this phenomenal happen also during chilling induced (Schoot *et al*., 2001). Glucose-6-phosphate dehydrogenase (G6PDH) and 6-Phosphogluconate dehydrogenase (6PGDH), both PPP enzymes, showed increased trend in yam tubers and turmeric rhizomes and even before the visible appearance of sprouting (Panneerselvam *et al*., 2007). However, Wang *et al*. (1991) showed a decrease in G6PDH and 6PGDH activity during budbreak and resumption of growth in lateral apple buds treated with Thidiazuron. Linsley-Noakes (1989) correlated the increase of proline in Dormex™ treated with the activation of PPP, resulting in the highest percentage of bud break. In this experiment, the PPP in the control had no significant changes compare to the treated dormant buds (Fig. 3).

Regarding the cytochrome pathway, HC treated buds showed a higher respiration than the control (Fig. 4). This change in respiration is in agreement with Pérez *et al*. (2009) who stated that mitochondria may be a potential detector or dormancy release. A short inhibition in CYT was observed during the first week of HC application compared to the control. The CYT inhibition may be involved in the HC application, H_2O_2 production followed with dormancy release. Amberger (2013) reported the he hypoxia state created by HC and/or oil inhibition oxidises cytochrome and blocks oxygen uptake in the electron transport. In the same way, high level of CO_2 was found as a source of inhibition of respiratory enzymes (e.g., cytochrome c oxidase, succinate dehydrogenase) (Gonzalez-Meler *et al*., 1996). The anaerobic conditions induce fermentative metabolism, pyruvate decarboxylase and alcohol dehydrogenase enzymes act on pyruvate and produce ethanol, CO_2 and oxidise NADH; which Block for short time the aerobic respiration in mitochondria (Taiz and Zeiger, 2010), which induce a decline in cytochrome pathway (Gonzalez-Meler *et al*., 2004). Furthermore, CYT were activated rapidly after the inhibition compared to control. This might be a symbol of growth resuming, because the relative growth rate of plants is highly dependent on CYT activity (Florez-Sarasa *et al*., 2007).

The alternative pathway was not sensitive to the treatment, and was relatively stable in both treated and control dormant buds during the first week after treatment (Fig. 5). However our study results showed that Dormex™ and oil had an effect on ALT compare to control buds after the first week of constant activity. Alternative pathway activity was almost constant irrespective of the fact that growth rate appears to be involved in the maintenance of the respiration component and showed relatively high activity in both HT treatment and control most of the time (Florez-Sarasa *et al*., 2007). Previous studies suggested important role of ALT pathway in prevention of damage to the cell by over-reduction of the respiratory chain and result in the formation of harmful ROS (Yip and Vanlerberghe, 2001; Robson and Vanlerberghe, 2002). Vanlerberghe *et al*. (1995) considered alternative pathway to be a regulatory mechanism of environmental adaptation. Previous studies showed that stresses can lead to alternative pathway disparity (Millar *et al*., 1993).

This study was interested in changes in total respiration and respiration pathways of dormant 'Cripps Pink' apple buds, treated with HC and oil. The rest breaking agent inhibited bud respiration for a short time after application, while breaking dormancy. The inhibition in total respiration, TCA and CYT is a direct result of HC treatment; and their activation follows in

later stage that breaks dormancy. Therefore, PPP and ALT may play a role in HC- induced dormancy breaking.

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