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# Ethrel® stimulant increases the activity of soluble invertase isoforms in rubber tree (*Hevea brasiliensis*) bark tissues

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**Abstract**

Ethrel® is a commercial stimulant based on ethylene commonly used in exploitation systems for increases sucrose hydrolysis and improve dry rubber production by rubber tree (*Hevea brasiliensis*). The main enzymes that act on sucrose hydrolysis and provide hexoses for rubber biosynthesis are invertase isoforms: Vacuolar Invertase (VIN), Cell Wall Invertase, (CWIN) and Neutral Invertase (NIN). The aim of this study was to evaluate the contribution of each of the invertase isoforms (VIN, NIN and CWIN) in rubber tree bark tissues stimulated with Ethrel® and its relationship with the rubber production. For this, they were made two Ethrel® applications in commercial planting of RRIM 600 clone rubber trees, a 34-day interval. Ethrel® stimulated rubber trees showed a 40% increase in dry rubber production. This work demonstrated that this increase is due to the higher performance of the NIN and VIN invertase isoforms, which contributed to the availability of hexoses in the intracellular compartment favoring rubber biosynthesis and improving the inflow of water necessary to the latex flow.

**Keywords:** *Hevea brasiliensis,* Invertase isoforms, Ethrel® stimulation, Latex flow, Tapping
**Abbreviation:** CWIN_Cell Wall Invertase, ES_Ethrel® Stimulated, IPP _Isopentenyl diphosphate, NES_Not Ethrel® Stimulated, NIN_Neutral Invertase, RS_Reducing sugars, SUC_Sucrose, Susy_Sucrose synthase, TSS_Total soluble sugars, UDP_Uridine diphosphate, VIN_ Vacuolar Invertase, WC_Water content.

## Introduction

The rubber tree, belonging to genus *Hevea*, is a most important species of Euphorbiaceae Family. It is native from tropical regions, including Brazil's zones, and cultivation is justified mainly because is natural rubber producer, raw material widely used in tires production and large number of manufactured products (Imle, 1978).

Natural rubber is polyisoprene obtained from latex extracted of rubber tree bark injury. Its production is related to changes in climatic conditions, photo assimilates synthesis and transport, carbohydrates reserve availability and their partitioning between natural plant drains (roots, stems, branches, flowers and fruits) and artificial drainage created by tapping. Thus, economic rubber tree exploitation involves the partitioning of these photo-assimilates to growth points and metabolites supply to lactiferous system where latex biosynthesis is processed (Gomez, 1982). Latex consists in emulsion of polymers micro particles in aqueous medium and its biosynthesis is main metabolic characteristic of lactiferous vessels and requires exclusively sucrose as precursor (Tupý, 1985). In lactiferous vessels, sequence of reactions involving sucrose hydrolysis culminates in hexoses availability to glycolytic route, where there is pyruvate generation that will be converted by enzyme pyruvate dehydrogenase into Acetyl-Coenzyme A (Acetyl-CoA). Isoprene anabolism by mevalonate route, from acetyl-CoA, allows isopentenyl diphosphate (IPP) formation, rubber basic unit (Chow et al., 2012). However, latex production, as well as other secondary metabolites, may be affected by plant hormones, but among them only ethylene has been identified to stimulate this function in rubber trees (Zhu and Zhang, 2009). Ethrel®, whose active principle is ethephon (2-chloroethylphosphonic acid), is latex flow stimulant that remains stable in acid form, but when applied in tapping's cutting allows the evolution of plant hormone ethylene (Mongan, 1972). Rubber tree bark stimulation through Ethrel® is considered of great importance for rubber exploitation systems, since its effects are favorable to maintenance of high production levels (D'Auzac et al., 1989). The latex biosynthesis response to stimulation is measured by ratio between production of stimulated tapping panel and production of same panel without stimulation (Dusotoit-Coucaud et al., 2010). Several positive metabolic aspects of Ethrel® stimulation to improve latex flow and increase rubber productivity have been reported in recent years, e.g., ethylene released into bark tissues acts on membrane permeability, leading to prolonged latex flow through decrease in rate of lactiferous vessels obstruction and in general regenerative metabolism (Zhu and Zhang, 2009).

Maintains lactiferous vessels with more rigid and thickened walls by avoiding occlusion and inhibiting latex coagulation (Boatman, 1966). In addition, Ethrel® stimulation also increases sucrose hydrolysis in bark tissues of rubber tree, resulting in glycolysis acceleration and improving the carbon supply source for IPP biosynthesis (Tupý, 1985; Mesquita et al., 2006). The main enzymes involved in sucrose hydrolysis in drain tissues are sucrose synthase (SuSy) (EC 2.4.1.13) and invertase (EC 3.2.1.26) (Koch, 2004). SuSy catalyzed reaction involves cleavage of UDP-dependent sucrose, producing UDP-glucose and fructose (Winter and Huber, 2000). Study conducted by Cairo et al. (2009), SuSy activity was found to be relatively low in non-stimulated rubber tissue from Ethrel®, this may be related to high $K_m$ (40 to 200 mM) of the SuSy for sucrose or by limitation in concentrations UDP in cytosol (Avigard, 1982; Loef et al., 1999). Tupý and Primot (1982) observed that in Ethrel® stimulated trees there was an increase in latex pH which significantly decreased Susy activity in this material. Invertases convert sucrose to glucose and fructose (Sturm and Tang, 1999). There are at least three invertase isoforms in plant tissues, which differ from one another by subcellular location and properties (Sturm, 1999; Roitsch and González, 2004). Acidic invertases are in vacuole (soluble acid invertase) or associated with the cell wall (insoluble acid invertase) while the neutral (soluble neutral invertase) is in cytosol (Sturm, 1999). Several studies reports that invertase isoforms cooperate sucrose partitioning in drain tissues. In *Eriobotrya japonica* Lindl, the increase in vacuolar invertase (VIN) activity accelerated sucrose hydrolysis in developing tissues and positively affected plant growth (Wang et al., 2015); On the other hand, the cell wall invertase (CWIN) activity increase is associated with higher hexoses content in sugarcane internode tissue (Lingle 1989). In *Arabidopsis thaliana*, higher CWIN activity is considered an essential component in plant defense against pathogens (Zhao et al., 2015). Thus, it seems clear that sucrose hydrolysis distribution between invertase isoforms in different cell compartments influences physiological responses according to tissue demand. In rubber trees, different invertase isoforms activity are strongly related to the raw material (Acetyl-CoA) supply for latex biosynthesis. Although Ethrel® treatment is a stimulant of latex invertase activity (Jacob et al., 1982; Tupý, 1985; Yeang et al., 1986), to understand the sucrose metabolism regulation in lactiferous tissues is of most importance for studies related to improvement rubber productivity. However, the contribution of different invertase isoforms for hexoses availability in bark tissues of rubber tree in response to treatment with Ethrel® is not known. Therefore, with this study we aimed at was assess the participation of each of the different invertase isoforms (VIN, NIN and CWIN) in rubber tree bark tissues Ethrel® stimulated and its relationship with rubber yield in RRIM 600 clone.

**Results and discussion**

***Climatic conditions***

During the trial period, precipitation (7.07 mm) was lower than ideal conditions for cultivation of rubber trees, since optimal conditions associated with maximum temperature values of 30.4 °C - 22.8 °C and a minimum precipitation of 72 mm (Rao et al., 1998). From this perspective, physiological characteristics and latex production discussed in this study took into consideration atypical dry period that precedes and subsist throughout this work. Although no visual characteristics were observed in response to drought in the rubber trees evaluated in this study, low soil water content reflects veering in gas exchange due to stomatal closure, which is strategically activated to reduce water loss (Medrano et al., 2002). However, this strategy may limit assimilation of $CO_2$ photosynthetic and cause changes in carbohydrate metabolism that are essential for latex biosynthesis.

***Aspects related to dry rubber production and latex flow***

Rubber production physiology is unusual compared to other crops because economic product is obtained from injuring the tree and creating an artificially induced drain, which results in reduction of plant growth rate. This occurs because photo assimilates in leaves, especially sucrose, before directed to meet only the demands natural drains of plant became strongly directed to latex reconstitution extracted, as well as bark regeneration. Average values rubber production and clogging index of lactiferous cells for both application periods of stimulant Ethrel® are shown in Table 2. Rubber trees Ethrel® stimulated (ES) showed a 40% increase in dry rubber production compared to not Ethrel® (NES) stimulated rubber tree. Stimulation with Ethrel® has been an essential practice for rubber production increasing of worldwide (Lacote et al., 2010).

Production enhance of dry rubber in ES trees (Table 2) is consistent with results found in literature (Gorton, 1972; Zhu and Zhang, 2009; Tang et al., 2010; Dusotoit-Coucaud et al., 2010). It has been reported that bark treatment on rubber trees with Ethrel® induced a decrease in latex solid total concentrations (Coupé and Chrestin, 1989), which corresponded to an inflow of water in latex vessels (An et al., 2016; Tungngoen et al., 2009). Once which, mature lactiferous cells are plasmodesmata devoid (De Fay et al., 1989), it has been suggested that aquaporin could be a physiological mechanism that induces latex production increase by ethylene stimulation, because aquaporin activity is regulated at post-translational level in response to hormonal treatments (An et al., 2016). Work performed with PB217 clone confirmed the genes expression that regulate aquaporin transcription (*HbPIP2;1* and *HbTIP1;1*) was clearly promoted by ethylene, which resulted in increased yield latex dilution after treatment with Ethrel® (Tungngoen et al., 2009).

Another important aspect that contributes to latex flow is pressure turgor of phloem at time of drainage, because it is initial driving force for latex flow. Incision made for latex extraction causes exudation by contraction of these vessels, which promotes a reduction in turgor pressure and establishes a difference in water potential with surrounding tissues. As result, water transfer for lactiferous cells causes latex dilution (Buttery and Boatman, 1967), and this process is facilitated by increased aquaporin transcription.

Stimulation with Ethrel® is also related to increase in lutoides stability that are bipolarized membrane particles containing B-serum, which through rapid flocculation action of rubber particles is responsible for latex coagulation. Disruption of these particles is related to lactiferous cells obstruction (Gidrol et al., 1994). Thus, lutoides stability is

inversely related for obstruction index and is directly related for rubber production. Combination these events are in part responsible for latex flow prolongation observed in this study. For obstruction index of the laticifers in the tapping cut, it was observed that for NES trees, the latex flow was 55% lower than ES trees (Table 2). These data were according with results observed for dry rubber production in this study because the stimulation effect resulted in an increased latex exudation period. Through decrease in obstruction index, ES trees reflect production increase because greater latex flow occurred during tapping completion not only in first five minutes, but throughout entire latex flow time. In drain, latex flows from lactiferous cells in cutting direction in region called tapping section area. Rubber production per tapping section area was also increased after Ethrel® stimulation. Work performed by Frey-Wyssling (1933) and subsequently by Schweizer (1941) showed that drainage area was located almost entirely below cutting with two small zones located next to and above. Drainage area size and sucrose supply in tissues that composed it, were probably proportional the capacity of rubber production in response the stimulant applied directly tapping panel, since the reported effects latex production increase were intensified in this region.

***Water content, reducing sugars, total soluble sugars and sucrose***

Water content (WC) analyses, total soluble sugars (TSS), reducing sugars (RS) and sucrose (SUC) in bark tissues of rubber trees are shown in Table 3. RS content for ES trees was higher than in NES, unlike of observed for TSS which was lower in ES trees. Already, WC and SUC showed no significant differences between treatments. A lower sucrose content and a greater RS content near cutting regions are correlated for rapid sucrose utilization through its hydrolysis with purpose of making hexoses for latex biosynthesis (Tupý, 1985) as well as to mitigate the effects of low water availability, through of cellular osmotic potential reduction. In this study, it was observed that most of soluble sugars (TSS) were comprised of reducing sugars, which indicated that sucrose hydrolysis by invertase isoforms were present in that tissue which led increased hexose availability for glycolytic route and consequently enabled greater IPP production. Work with sucrose ($^{14}C$) application in tapping panel resulted radioactivity appearance in cytosolic fraction of latex that was collected, and process was enhanced by Ethel® stimulation (Eschbach et al., 1986).

In general, latex production in rubber tree bark is related to regeneration of new latex quantities and depends on frequency at which tapping is performed. Therefore, carbohydrates supplied to bark fabric and used in rubber biosynthesis must be sufficient to withstand high demands that will reflect physiological parameters related to production.

***Activity of invertase isoforms***

Was observed significant difference between treatments NES and ES for VIN activity in all days evaluated (Fig. 2). Regarding effect of trial period under both treatments, only at 21 days after first stimulation VIN activity was statistically lower in ES trees. For NES trees, it was observed that at 7 days after first application of stimulant the activity of VIN was higher than other days analyzed.

Higher VIN activity in both treatments was found for the lower precipitation index observed in the trial period. increase VIN activity is an important player for hexose accumulation in vacuoles, which function as osmotically active solutes and contributed to mitigating effects of water shortages caused by low rainfall recorded. Biochemical processes are dynamic and are affected by endogenous as well as environmental factors, especially in conditions with low water availability, which cause a decrease in cell volume and promote sugars accumulation (Davies, 1996; Santos et al., 2013). Metabolites accumulation in plants under drought conditions provides an osmotic potential reduction by net increase in vacuolar solutes concentration (Kavas et al., 2015). This was verified in this work, when TSS and RS (table 3) increased significantly in ES trees. This adjustment helped rubber tree to maintain turgor and water inflow in inner bark tissues, as well as contribute to and extend latex flow. Thus, VIN activity increase has positive relationship with low rainfall recorded in region as well as the Ethrel® stimulation because higher values were obtained from ES trees.

Studies have revealed that bark treatment with Ethrel® activates ATPase connected to tonoplasto vacuole (Gidrol et al., 1988), translocating cytosol protons to this compartment and thus favors tonoplasto acidification. Alkalinizing pH in cytosolic metabolic processes, improves sucrose catabolism intracellularly and lactiferous cells regeneration through multiple activation dependent enzymes, mainly to NIN, since this isoform working best in alkaline pH (Jacob et al., 1989). VIN promotes sucrose cleavage at greater efficiency when exposed to pH levels between 4.5 and 5.0, and NIN activity is maximized at pH levels between 7.0 and 7.8 (Sturm, 1999). Thus, increased ATPase activity linked for tonoplasto membrane promotes positive response for stimulation with Ethrel®, both through VIN by vacuoles acidification as well as NIN through cytosol alkalization.

NIN activity, shown in Fig. 3A, was higher in ES trees for both periods of stimulant application. Larger increments, were observed in analyzes made soon after stimulant applications in both periods (7 and 12 days). In same way as VIN activity (Fig. 2), NIN activity at 21 days presented a reduction when effect of trial period between treatments was compared. In NES trees time did not influence performance of this isoform.

NIN activity in ES trees exceeded NES trees activity by 37%. However, in both Ethrel® stimulation the hormonal effect dropped with time passage, although stimulated trees had a higher activity NIN compared to NSE trees. In studies on effect Ethrel® stimulation in PB217 clones performed by Dusotoit-Coucaud et al. (2010), in virgin mature trees, it was found that increased latex flow with stimulation occurred for early tapping, which was 27 times higher than control (not stimulated trees) at 40 hours after treatment reducing activity over time.

Similarly VIN, NIN activity also has performance favored by increased ATPase activity in this case, bound to plasma membrane and is promoted by stimulation with Ethrel® through symporter system intensified sucrose/H+ transport being involved in sugars absorption in lactiferous cells (Bouteau et al., 1992). Studies using carbon isotopes showed

**Table 1.** Panel characteristics of evaluated rubber trees in this work. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Means were compared by Tukey test at 5% probability (p<0.05). CV: coefficient variation and SE: Standard error.

| Treatments | Cutting length cm | Trunk girth cm | Bark thickness cm | Tapping section area $cm^2$ |
|---|---|---|---|---|
| NES | 36.0* | 58.2* | 0.5* | 21.1* |
| ES | 36.1 | 58.1 | 0.6 | 22.3 |
| VC | 5.75 | 5.76 | 19.06 | 24.64 |
| SE | 0.51 | 0.83 | 0.02 | 1.33 |

*Averages do not differ in column.

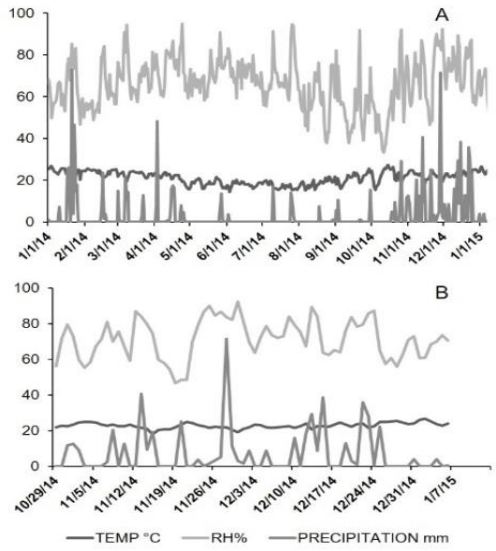


**Fig 1.** The graphic shows the fluctuation of temperature, relative humidity and precipitation, during the year 2014 and January 2015, where the experiment was carried out. 4B represents the period in which the data were collected (October 2014 to January 2015) within a mean rainfall range of 4.15 to 7 mm at 7 and 21 days; 9.4 mm from 21 to 50 days and 5.3 mm from 50 days to 70 (end of trial period).

**Table 2.** Dry rubber production and obstruction index of laticifers vessels. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Case letters compare treatments NES and SE. Means were compared by Tukey test at 5% probability (*p<0.05*). VC: variation coefficient and SE: Standard error.

| Treatments | Dry rubber production ($g.tree^{-1}.tapping^{-1}$) | Dry rubber production ($g.cm^{2}.section\ area.tapping^{-1}$) | Laticifers vessels obstruction % |
|---|---|---|---|
| NES | 38 b | 1.9 b | 14.5 a |
| ES | 53 a | 2.7 a | 8 b |
| VC | 28.38 | 19.85 | 21.44 |
| SE | 4.78 | 0.31 | 1.14 |

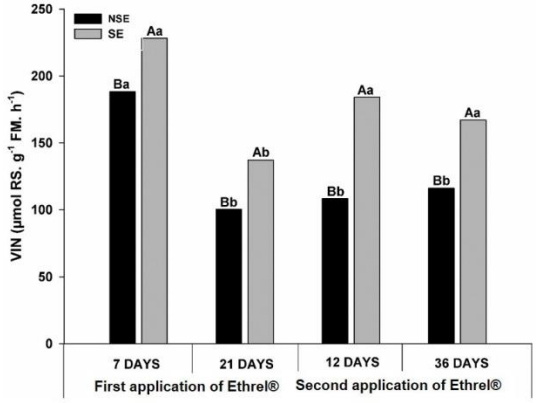


**Fig 2.** Vacuolar invertase (VIN) activity in bark tissues of rubber tree for two periods of Ethrel® stimulation (first and second application). RS: reducing sugars. FW: fresh weight. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Averages, was compared by Tukey's test at 5% probability level ($p$<0.05). Capital letters compare the treatments at each time of evaluation and lower-case letters compare the treatments throughout trial period.

**Table 3.** Water content (WC), total soluble sugars (TSS), reducing sugars (RS) and estimated sucrose content (SUC) in dry matter (DM) of bark tissues of rubber trees. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Case letters compare treatments NES and SE. Means were compared by Tukey test at 5% probability ($p$<0.05). VC: variation coefficient and SE: Standard error.

| Treatments | WC (%) | µmol TSS.g$^{-1}$ DM | µmol RS.g$^{-1}$ DM | µmol SUC.g$^{-1}$ DM |
|---|---|---|---|---|
| NES | 36.0* | 1022.37 b | 477.78 b | 544.59* |
| ES | 36.4 | 1164.77 a | 602.08 a | 562.68 |
| VC | 1.75 | 5.08 | 8.43 | 5.03 |
| SE | 0.23 | 13.89 | 11.38 | 6.95 |

*Averages do not differ in column.

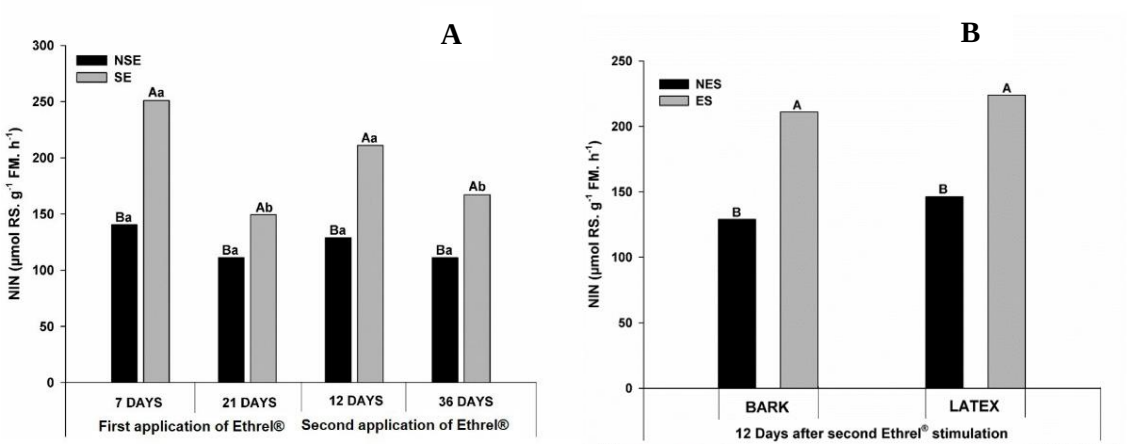


**Fig 3.** A- Activity Neutral Invertase (NIN) in bark tissues of rubber tree for two periods of Ethrel® stimulation and B- Comparison between activity Neutral Invertase in bark tissue and in latex collected at 12 days after second stimulant application (first and second application). RS: reducing sugars. FW: fresh weight. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Averages was compared by Tukey's test at 5% probability level (p<0.05). In fig. 3A, capital letters compare treatments NES and ES at each time of evaluation and lower-case letters compare treatments throughout trial experimental. In fig. 3B, capital letters compare treatments NES and ES in analyzed material.

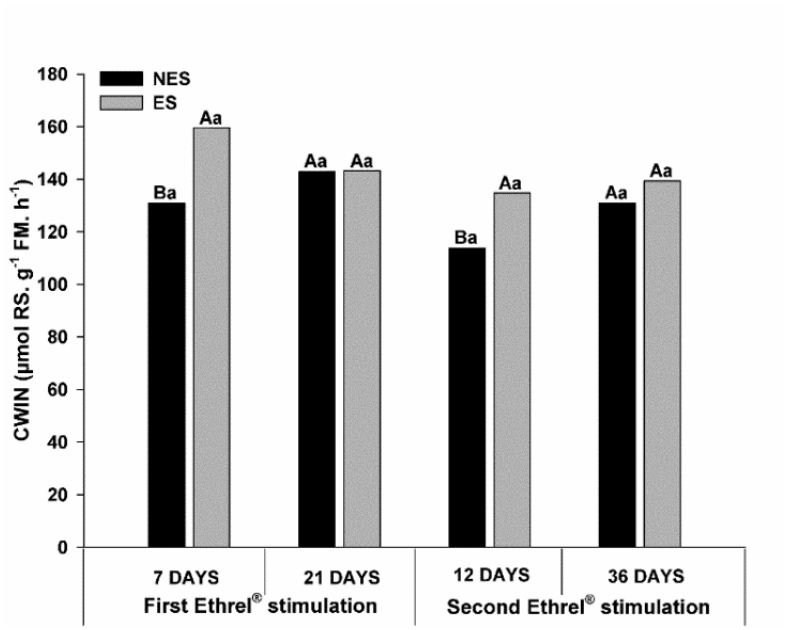


**Fig 4.** Activity Cell wall Invertase (CWIN) in bark tissues of rubber tree for two periods of Ethrel® stimulation (first and second application). RS: reducing sugars. FW: fresh weight. NES: Not Ethrel® stimulated; ES: Ethrel® stimulated. Averages, was compared by Tukey's test at the 5% probability level ($p$<0.05). Capital letters compare treatments at each time of evaluation and lower-case letters compare treatments throughout trial period.

that sucrose had high affinity for symporter with H+ across plasma latex vessels membrane, this characteristic showed high capacity in this drain for importing that sugar (Tang et al., 2010). Stimulation with Ethrel® could also enhance NIN activity through sucrose transporters increase. Sucrose transporters play an important role in phloem loading and sucrose unloading, these transporters participate in transferring between apoplastic and simplastic compartments (Chen et al., 2012; Tang et al., 2010; Braun and Slewinski, 2009; Riesmeier et al., 1994).

Work with sucrose transporters have shown that only two (*HbSUT1A*, *HbSUT2A*) were found to be significantly stimulated by ethylene (Dusotoit-coucaud et al., 2010) and promote greater sucrose supply to intracellular environment of lactiferous cells (Coupé and Chrestin, 1989; Zhu and Zhang, 2009). Availability sucrose transporters in *Hevea* bark tissue may be possibly involved in increase soluble invertase activity (VIN and NIN), which would facilitate entry of substrate of apoplast to cytosol as well as from cytosol for vacuoles. Important factor that must be considered is fact that all mechanisms mentioned in this study with explain high NIN activity in bark tissues, can also promote sucrose synthase (SuSy) activity because both enzymes have same cellular location. Despite being involved in hydrolysis of same substrate (sucrose), participation these enzymes in various processes that lead to growth and plants development occurs differently (Cairo et al., 2009). High SuSy $K_m$ for sucrose and limitations imposed on this enzyme by cytosol UDP concentrations (Avigard, 1982) do not totally prevent activity have been superimposed on NIN activity. Tupý and Primot (2008) observed that under natural conditions, sucrose cleavage by SuSy is significant especially in bark regeneration. However, the same authors reported that the Ethrel® stimulation increased the rapid sucrose use and the rubber production by raising the pH, which favors NIN activity while it cannot be related to SuSy activity (Sturm and Tang, 1999).

Other evidence suggested that alterations in mineral nutrition influence activity of some enzymes (Jacob et al., 1982; Prado et al., 1979) because activators such as phosphates, nitrates and thiols may be present in concentrations cytosolic sufficient to promote alkalization in this compartment and influence NIN activity through sucrose hydrolysis, which is key step in latex biosynthesis. However, there have be no studies correlating Ethrel® stimulation and mineral nutrition on invertase isoforms activity in rubber trees. It is also necessary to consider that the mevalonate pathway occurs in the cytosolic compartment and can be directly stocked with Acetyl-CoA produced through the sequential steps of sucrose hydrolysis, in this compartment is reinforced by NIN activity.

In this study, NIN activity was evaluated in latex can be explained by fact that latex is essentially cytoplasmic contents of lactiferous cells (De Fay, 1989; Chow et al., 2012). In Fig. 3B, there is a comparison between NIN activity analyzed in bark tissues and latex from samples collected at 46 days into work, corresponding to 12 days after second Ethrel® stimulation.

NIN activity in latex increased significantly after stimulant applying. However, there was no difference in material type that was analyzed, clearly showing that cytosolic isoform activity analyzed in latex could be used to estimate NIN activity in bark tissues because they have same values. It is also necessary to consider that the mevalonate pathway and IPP synthesis occurs in the cytosolic compartment and can be directly stocked with Acetyl-CoA produced through the sequential steps of sucrose hydrolysis, which in this compartment is result of NIN activity. Higher productivity observed in this study for ES trees, is positively related to performance of this enzyme.

In Fig. 4, it was found that CWIN activity in ES bark tissues responded positively to treatment only in first evaluation performed after the two times of stimulant application (7 and 12 days after first and second Ethrel® stimulation, respectively). However, when compared influence of treatment over trial period, were not observe statistical differences. Despite the increase in evaluations made shortly after both stimulant applications, increased CWIN activity (Fig. 4) was lower when compared to intracellular isoforms (VIN and NIN), this could be attributed to the fact that this enzyme (CWIN) was substantially saturated with the substrate (sucrose). These intracellular isoforms intensification is related to increase in sucrose transporter expression influenced by Ethrel® stimulation (Dusotoit-Coucaud et al., 2010), facilitating the transfer this sugar between apoplastic and simplastic compartments.

**Materials and Methods**

***Plant material***

Activities in laboratory and field conditions were conducted using an existing planting of eight-year-old rubber trees in crop production phase in Nepomuceno, Minas Gerais, Brazil. Geographic coordinates were 21°17’33’’S latitude, 45°10’41’’W longitude, and altitude was 904 m. During trial period - 72 days, relative humidity values of air varied from 92.25% to 46.5%, average temperature was 22.4 °C, and average rate precipitation was 7.07 mm. However, 2014 year in which we carried out this work, was characterized by one of worst water crises to hit large areas of Brazil, including Minas Gerais State, where rainfall index (7.07mm) at the site of this study was significantly below of minimum volume expected for rainy season (on average 620 mm). The climatic data for experiment site can be seen in Fig. 1.

***Experimental conditions***

Experimental treatments were composed this way: rubber trees that received Ethrel® stimulation (ES) and not Ethrel® stimulated (NES). Ethrel® (Produced by Bayer S/A, registered in Ministério da Agricultura, Pecuária e Abastecimento/ MAPA sob nº 01505), was dissolved in 1 L of water (0.5% w/v).

Ethrel® solution and applied with brush in drainage channel following the manufacturer's technical recommendations for product use. Each stimulated tree received approximately 0.5 mL of solution. A second Ethrel® stimulation occurred at 34 days after first in same trees selected for first stimulation. In total, there were 18 latex collection, and each stimulant application corresponded to nine tapping.

***Dry rubber production and aspects related to latex flow***

Latex collection system, was half-spiral (½ S) in inclination of 35°, with 2 collections per week spaced between three to four days (3/d and 4/d), taken at approximately 1.2 meters from soil surface. Collections were made between 7 and 9 am, and latex coagulations were pressed and put up in dry place, covered and ventilated at medium temperature of 28 °C. Rubber content was collected from final production of 18 rubber tree tapping. Dry rubber contents were weighed after 28 days to obtain production data (Table 2). Table 1, show measures cutting length, trunk girth, bark thickness and tapping section area. There was uniformity among selected trees for this study in terms of panel features. As such, these features influence productivity in rubber tree clones. To obtain cutting length and tapping section area, some calculations were performed as shown below:

$$\begin{aligned} &Cutting\ Length \\ &= Trunk\ girth\ .\ tapping\ system\ (½\ spiral) \\ &/ \cos 35°\ (tapping\ panel\ slope) \\ &\qquad Section\ length \\ &= trunk\ radius\ .\cos 35°\ (tapping\ panel\ slope) \\ &Tapping\ section\ area = Section\ length\ .Bark\ thickness \end{aligned}$$

Dry rubber yield data were obtained from medium tapping performed during this work (18 latex collection). Dry rubber production (g) per $cm^2$ of tapping section area also was estimated. Obstruction index of laticifers (%) vessels was obtained between 7-8 am, by measuring latex amount drained in first 5 minutes after latex flow is started, and dividing by total latex volume collected at tapping end and result was multiplied by 100. Obstruction index evaluation was conducted in 5 NES and 5 ES replicates at 7 and 12 days after first and second Ethrel® stimulation respectively.

***Bark samples and biochemical analysis***

All plant materials used for biochemical analyses were obtained by cutting performed on panel of 32 rubber trees (16 NES and 16 ES) with aid of a knife for tapping between 8 and 9 am at 7 and 21 days after first Ethrel® stimulation, as well as at 12, and 36 days after second stimulation. Two bark strips, approximately 1 mm thick, were removed; first was discarded and second was wrapped immediately in liquid nitrogen and transported to laboratory where samples were transferred to storage in a freezer at - 80°C until analysis.

Water content (WC), total soluble sugars (TSS), reducing sugars (RS) and sucrose (SUC) were estimated in composed of 16 biological replicates analyzed in technical triplicate. Samples were composed of 5 g of fresh weigth, referent at mix of four samplings obtained during the work. WC was determined by gravimetric method (AOAC, 2000). TSS were determined by reaction with anthrone (Hodge, 1962). Additionally, RS were determined according to methodology described by Nelson (1944). Sucrose content was estimated by difference between TSS and RS.

For invertase isoforms activity evaluation, was using 100 g fresh weight, composed of 16 biological replicates analyzed in technical triplicate. Protein extract was obtained as described by Cairo et al. (2009), and it was used as crude source of enzymes. Supernatant was used for carrying out enzymatic assay of neutral invertase from cytosol (NIN) and acid invertase from vacuole (VIN) and pellet for extraction of cell wall invertase (insoluble invertase), which was performed according to Fahrendorf and Beck (1990) with modifications. Pellet was resuspended in 0.1 M sodium acetate buffer at pH 3.5, 0.5 M MgCl2, 0.2 M sucrose, 1 M NaCl and Triton-X (1%). Subsequently, solution was centrifuged at 18.000 g at 4 °C for 20 minutes and supernatant was used for carrying out enzymatic assay. Reaction medium (1.5 mL) was composed of 0.1 M sodium acetate buffer pH 3.5, 0.5 M MgCl2, 0.2 M sucrose and 200 µL protein extract aliquot. For latex neutral invertase extraction, 20 mL of latex was obtained 12 days after second Ethrel® stimulation. Material was centrifuged at 18.000 g at 4 °C for 1 hour for C-serum extraction. After extraction, aliquot of 300 µL of C-serum was added to 4 mL of reaction medium containing 2.1 mL of 0.2 M potassium phosphate buffer, pH 7.4, 2 mL of 0.3 M sucrose and 1.4 mL of distilled water. Solution was then incubated in water bath at 30 °C. In all of described assays, the invertase isoforms were quantified through aliquots of 100 µL of reaction medium collected at the end of 10 and 70 minutes of incubation for quantification reducing sugars using DNS method, Miller (1959).

***Experimental design***

Experimental design used was entirely randomized blocks with 2 treatments, each with 16 replicates, adding 32 rubber trees. Data were submitted to analysis of variance (ANOVA) using the statistical software SISVAR 4.3 (System Analysis of Variance for Balanced Data, Lavras, Brazil) (Ferreira, 2014).

**Conclusion**

Among the reported effects to improve rubber yield in rubber trees, it was found Ethrel® stimulant (0.5 mL of solution 0,5%) applied on interval of 34 days, decreases rate of lactifers vessels obstruction, prolonging latex flow and increasing dry rubber production (40%) in RRIM 600 clone. This increase is mainly explained by greater sucrose hydrolysis in bark tissues promoted by increased activity of cytosolic and vacuolar invertase isoforms (NIN and VIN), what is compatible with greater carbon availability for isopentenyl diphosphate production in cytosolic compartment and greater water influx to latex flow respectively.

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***Conflict of interest***

The authors declare that they have no conflicts of interest.

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