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Effects of Gibberellic Acid (GA_3) on Strawberry PAL (Phenylalanine Ammonia-Lyase) and TAL (Tyrosine Ammonia-Lyase) Enzyme Activities

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Abstract: Gibberellic acid (GA_3) treatments (30 and 60 μg litre^{-1}) were applied to young plants (*Fragaria ananassa* cv Chandler). Fruits were harvested at various developmental stages (14, 21, 28 and 35 days from fruit set). Weight and size, phenolic compounds (total polyphenols and anthocyanins) and enzyme activities, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) were determined. Our aim was to obtain detailed information about PAL and TAL activities related to the strawberry colour during development and ripening processes and to determine the effects of exogenous treatments of GA_3 on PAL and TAL activities. Exogenous treatments of GA_3 improve weight, size and colour of strawberry fruits, and affect PAL and TAL activities. We found that the anthocyanin content and PAL activity are enhanced by the exogenous treatment of GA_3 in the range of 30 μg litre^{-1}. However, with the higher GA_3 treatment, only the anthocyanin content is affected in that way. These findings suggest that gibberellic acid effect on PAL, TAL and ultimately anthocyanin enhancement is dosage related and saturation of the response occurs at 30 μg litre^{-1}. © 1998 SCI.

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Key words: strawberry (*Fragaria ananassa*); gibberellic acid; PAL (phenylalanine ammonia-lyase); TAL (tyrosine ammonia-lyase); ripening; polyphenols; anthocyanins

INTRODUCTION

Strawberry (*Fragaria ananassa*) ripening is generally accompanied by simultaneous changes in colour (which involve loss of chlorophyll leading to the unmasking of underlying pigments and the synthesis of new pigments), flavour (which include changes in acidity, astringency and sweetness, themselves dependent on the organic acids, phenolics, sugars and volatile present in the tissue) and texture in normal fruit. Although the levels of phenolics in the fruit are relatively low, they can be quite significant compared to many other plant tissues, especially in determining the quality of fruit products (Tucker 1993).

It is well known that many phenolics are derived from phenylalanine via cinnamic and coumaric acids (Tucker 1993). Anthocyanin accumulation is a readily visible and easily measured change in ripening strawberry, a non-climacteric fruit. Red colour in strawberries originates from two main anthocyanin pigments, cyanidin-3-glucoside and pelargonidin-3-glucoside (Wesche-Ebeling and Montgomery 1990; Kalt *et al* 1993). In general, fruit colour change is associated with ripening and represents an attribute, along with texture, for the determination of its acceptability. Anthocyanins are derived from flavonoid compounds and as such are synthesised from the aromatic amino acid phenylalanine. Phenylalanine ammonia-lyase (PAL) is one of the

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key enzymes in controlling anthocyanin biosynthesis from phenylalanine, and it is known to be synthesised *de novo* in many plant tissues in response to UV light and mechanical damages (Tucker 1993).

PAL activity has been previously studied by Given *et al* (1988) and Cheng and Breen (1991), during development and ripening of strawberry fruits. However, tyrosine ammonia-lyase (TAL), another enzyme involved in phenolic synthesis, has not been explored in strawberry. Changes of this enzyme (TAL) and of the phenolic substances accompanying chilling-injury were studied by Kozukue *et al* (1979) in eggplant fruit. In addition, the activities of PAL and TAL following exogenous treatments of gibberellic acid have not been studied in strawberries before.

Little is known about the role of the gibberellic acid on fruit ripening. McGlasson *et al* (1978) suggested that these substances have an important incidence on the biosynthesis of the anthocyanins. We have only found in the literature studies of the use of hormones (auxin and cytokinin) to regulate anthocyanin production and composition in suspension cultures of strawberry cells (Mori *et al* 1994). Martínez *et al* (1994), using strawberry slices, studied the effect of gibberellic acid, GA_3, on colour development and on respiration activity. Their results showed that GA_3 had an inhibitory effect on strawberry ripening, evidenced by a decrease in the respiratory activity and a delay in anthocyanin synthesis and chlorophyll degradation. Applications of GA_3 on strawberry plants can advance the earliness of the crop, but the response is dependent on both climatic conditions and the cultivar grown. No negative effect was induced by the application of GA, in either total yield or fruit size (López-Galarza *et al* 1989). In addition, GA_3 has been recommended in other fruits (plum), to produce better colour and larger fruits with more firmness (Boyhan *et al* 1992).

In the present paper, we report investigations of enzyme activities responsible for the strawberry colour during development and ripening and the effect of exogenous treatments with gibberellic acid (GA_3) on weight and size of strawberry fruits, and also the possible relationship between them.

MATERIAL AND METHODS

Plant material

Strawberry fruits (*Fragaria ananassa* cv Chandler) were obtained from a greenhouse hydroponic system having controlled nutrition and automated temperature, humidity and lighting controllers. Temperatures ranged from 30°C during the day to 18°C at night. The relative humidity was kept between 65 and 80%. Daily irradiancy was 240 W m^{-2}. The experimental design was a randomised block system with series of nine pots containing three plants each.

Spray treatments of GA_3 were applied at the rate of 30 and 60 μg $litre^{-1}$, respectively (Fengib, Inagra, Valencia, Spain). The concentration of the product used was 0·5% (w/v) of GA_3. A tensioactive (Inagra) was added to the solutions (0·5 ml $litre^{-1}$) to improve the adherence of the spray to the plants. Sprays were applied to young plants without flowers and fruits at 3 months after planting runners in the hydroponic culture. Control plants (untreated) were sprayed only with distilled water and tensioactive.

Fruits were harvested at various developmental stages (14, 21, 28 and 35 days from fruit set). Fruit age was monitored by labelling with different colours the flowers obtained in each flowering, and counting the days after anthesis. The time period between the application of the sprays and the first fruit harvest was 1 month. Treated fruit started flowering 1 week before the control. Each particular sampling day included all the fruits (primary, secondary, etc) obtained in the different flowerings of the plants; each day, all the fruits were cut into pieces and well mixed to achieve representative results in all the assays performed. Fruits were weighed and sized (maximum length and diameter) without calyx each sampling day, and after that they were cut, mixed and frozen in liquid N_2.

Phenolic compounds

The fruits (10 g) were homogenised with 30 ml of extractant (methanol/formic acid, 1 : 0·05) and after 3 days of extraction (changes of extract every day), the supernatant was vacuum filtered. The liquid obtained was partly evaporated to obtain a final volume of 50 ml. Aliquots of the supernatant were used to determine the total polyphenols and the anthocyanins contents. Determinations of total polyphenols were made according to the Folin-Ciocalteu's method, using gallic acid standard (Montero *et al* 1996). Anthocyanin content was determined colorimetrically based on colour change at pH 3·5, using a standard of cyanidin chloride (Esteban *et al* 1989). Gallic acid was obtained from Sigma (St Louis, MO, USA) and cyanidin chloride from Extrasynthèse (Genay, France). Gallic acid and cyanidin-3-glucoside were prepared in the same solvent as the samples.

Enzyme preparation

The method of Cheng and Breen (1991) with some modifications (vacuum filtration instead centrifugation in the first steps of the extraction, and the use of the centrifuge at the end of the enzyme extraction) was used. Using a mortar and pestle, 10 g fruits were homogenised in 100 ml of cold acetone and the in-

soluble residue filtered and dried under vacuum. It was extracted at 4°C by gentle stirring with 50 ml of extraction buffer which contained 100 mM sodium borate (pH 8·8), 5 mM β-mercaptoethanol, 2 mM EDTA (ethylendiamintetraacetic acid) and PVPP (polyvinylpolypyrrolidone) at 100 g kg^{-1} of the fruit fresh weight. After 1 h of extraction, the solution was filtered through one layer of nylon cloth and centrifuged at 20 000 × g at 4°C for 15 min.

PAL and TAL activities

PAL and TAL activities in the buffer supernatant were determined by the production of *trans*-cinnamic and *p*-coumaric acids, respectively, from L-phenylalanine (PAL) and L-tyrosine (TAL), during 1 h at 30°C, as measured by the absorbance change at 290 nm (PAL) and 333 nm (TAL) (Kozukue *et al* 1979; Cheng and Breen 1991).

The assay mixture contained 1·5 ml 30 mM sodium borate buffer (pH 8·8) and 0·5 ml buffer supernatant, and 1 ml 0·015 M L-phenylalanine for PAL assays and 1 ml 0·015 M L-tyrosine for TAL assays. The substrates (L-phenylalanine for PAL and L-tyrosine for TAL) were added after 10 min of preincubation and the reactions stopped with 0·1 ml 6 M HCl. Assays were performed in triplicate. The molar extinction coefficient of authentic *trans*-cinnamic acid in assay buffer plus HCl was determined to be 19 207, and the molar extinction coefficient of authentic *p*-coumaric acid in assay buffer plus HCl was determined to be 6886. L-Phenylalanine, L-tyrosine, *trans*-cinnamic and *p*-coumaric acid were obtained from Sigma (St Louis, MO, USA).

Statistical analysis

All determinations were carried out in triplicate and expressed on a fresh weight basis. Data were subjected to analysis of variance and Duncan's test ($P > 0·05$) to determine the significance of differences between treatments (control, $GA_3(1)$ and $GA_3(2)$), and between the sampling days (14, 21, 28 and 35) of each studied parameter, by calculating the least significant differences at the 5% level (LSD). The units of the LSD obtained are the same of the particular parameter studied.

RESULTS AND DISCUSSION

Figure 1 exhibits the effect of GA_3 treatments on weight, length and diameter of the strawberry fruits (control and treated). As reported in the literature, the growth of the strawberry fruit follows a single sigmoid growth curve (Hartman *et al* 1981). Weight (Fig 1(a)) is parallel in the three treatments (control, $GA_3(1)$ and $GA_3(2)$), increasing steadily from the first stages of the

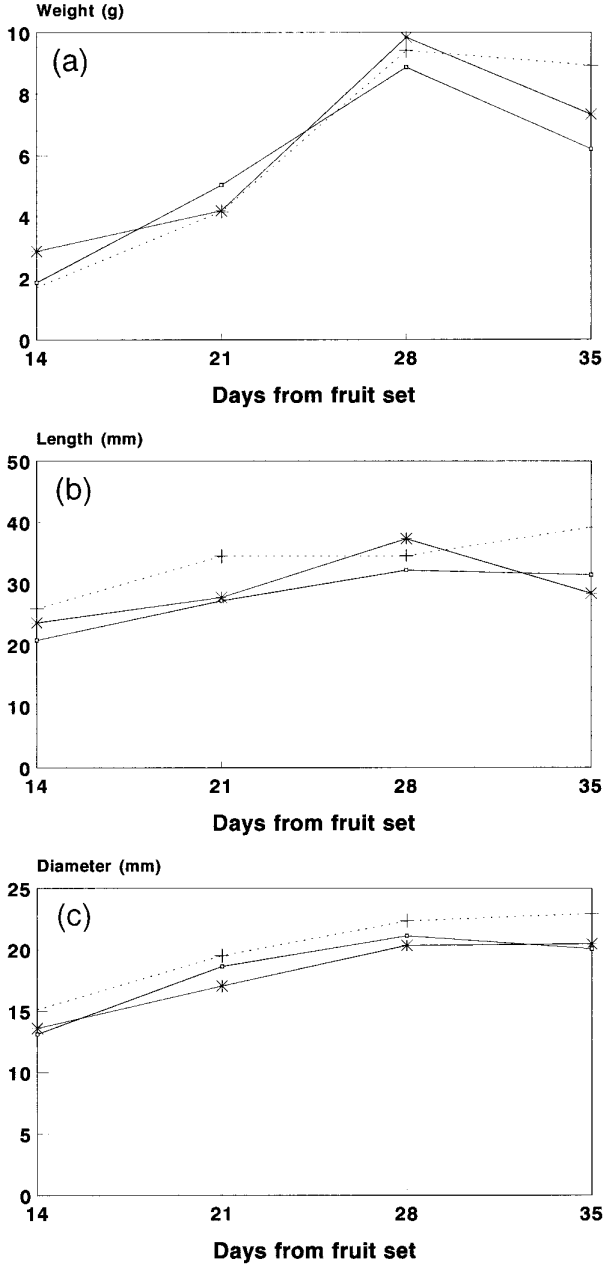

Fig 1. Effects of giberellic acid treatments on (a) weight (LSD(treatments)5% = 0·44, LSD(samplings)5% = 0·88; (b) length (LSD(treatments)5% = 1·47, LSD(samplings) = 2·94); and (c) diameter (LSD(treatments)5% = 1·03, LSD(samplings)5% = 2·07) during ripening strawberry fruits (control — □ —, $GA_3(1)$ ··· + ···, $GA_3(2)$ — ∗ — treatments)

study to ripeness and then decreasing until senescence, probably due to a noticed loss of water that occurs at senescence stages of the fruit, as appears in other fruits (Martín-Cabrejas *et al* 1994). Control and treated fruit reached the maximum weight at the same time, 28 days from fruit set. However, fruit treated with $GA_3(1)$ or $GA_3(2)$ showed a greater weight gain between 21 and 28 days from fruit set than the control. Control and fruit treated with 60 μg $litre^{-1}$ reach a maximum length by 28 days from fruit st (Fig 1(b)), while fruit treated with 30 μg $litre^{-1}$ reach a maximum length earlier (by 21

days from fruit set) which remained constant until 28 days. The diameter (Fig 1(c)) increased continuously from 14 to 35 days, the $GA_3(1)$ treatment causing the highest values, with significant differences during all periods studied. The maximum diameter obtained by treatment $GA_3(1)$ was between 28 and 35 days. Summarising, treatment $GA_3(2)$ reaches the maximum weight and length at 28 days from fruit set, but the highest diameter is obtained by $GA_3(1)$.

For all treatments, total polyphenols decreased rapidly with development, reaching a constant level at 28 days (Fig 2(a)). The high concentration of phenolics in young fruits is a common feature in strawberry cultivars (Spayd and Morris 1981). The polyphenol content of fruits treated with gibberellic acid is lower than the control, $GA_3(1)$ showing greater differences than $GA_3(2)$. These results agreed with those found by Cheng and Breen (1991). Total anthocyanins are shown in Fig 2(b). In untreated fruits (controls), anthocyanins were undetectable during the first stages of development until 21 days from fruit set, and then began to accumulate significantly until the 28th day, and reached a maximum level at 35 days. This trend is similar to that obtained by Cheng and Breen (1991), who did not detect anthocyanins until 23 days after anthesis. Fruit treated with GA_3 began to accumulate anthocyanin at 21 days. Fruit treated with 60 μg $litre^{-1}$ GA_3 reached maximum anthocyanin levels at 28 days, one week earlier than control fruit. Thus, strawberries treated with gibberellic acid $GA_3(2)$ show higher levels of anthocyanin than those of control.

In addition, the pattern of PAL activity (Fig 2(c)) differs from control to treatments. Control fruit patterns shows a decrease, without significant differences, in the enzyme activity from the first stages of development to 21 days from fruit set, and then there is an increase to reach a peak at the 28th day, when another starts decrease. Unlike Cheng and Breen (1991), who found two maximum peaks of PAL activity occurring during strawberry fruit development, we found only one such peak. This corresponds to the second peak of Cheng and Breen (1991), and occurs in control fruit a week before anthocyanin accumulation. $GA_3(1)$ treated fruits exhibited a pattern of PAL activity similar to that obtained from the control, although the enzyme activity was always higher in treated fruit. $GA_3(2)$ treated fruits showed similar but non-significant changes in PAL activity. The PAL activity of fruit treated with 60 μg $litre^{-1}$ was higher than control until 21 days from fruit set, then it was lower than other treatments.

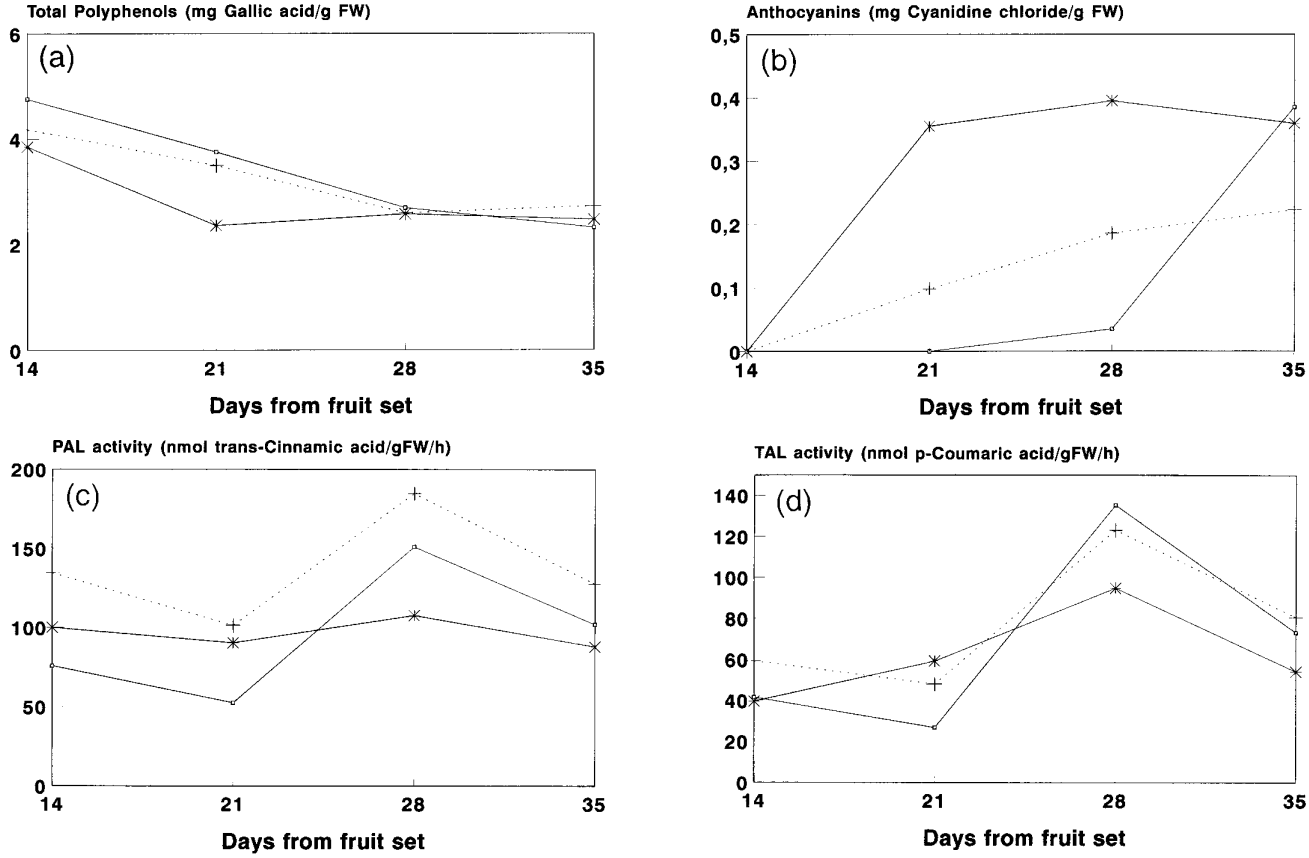

Fig 2. Effects of giberellic acid treatments on (a) total polyphenols (LSD(treatments)5% = 0·19, LSD(samplings)5% = 0·38); (b) anthocyanins (LSD(treatments)5% = 0·01, LSD(samplings)5% = 0·03); (c) PAL activity (LSD(treatments)5% = 9·290, LSD(samplings)5% = 18·58); and (d) TAL activity (LSD(treatments)5% = 5·41, LSD(samplings)5% = 10·83) during ripening strawberry fruits (control — □ —, $GA_3(1)$ · · · + · · ·, $GA_3(2)$ — ∗ — treatments).

In relation to TAL activity (Fig 2(d)), the trend is parallel to that obtained from PAL. However, TAL values are lower than those of PAL, consistent with other works on fruits such as eggplants (Kozukue *et al* 1979). Comparing control to treatments, the highest TAL activity is obtained by the control and treatment $GA_3(1)$, without significant differences, at 28 days from fruit set. $GA_3(1)$ TAL activity is higher than control until 21 days, being lower at 28 days. However, $GA_3(2)$ TAL activity is generally lower than control except around the 21st day from fruit set. In general terms, TAL activity follows the same pattern as PAL activity.

Therefore, this study shows a parallelism between the trend of anthocyanins and the enzymatic activities in control fruits (Fig. 2). Thus, in this work we demonstrate that an increase in anthocyanin content is accompanied not only by increases in the activities of PAL, but also by increases in TAL activities, and this is the first time that TAL enzyme is studied in strawberry fruits. Moreover, the results obtained about PAL activities are in agreement with those obtained by Given *et al* (1988) and Cheng and Breen (1991).

Summarising, $GA_3(1)$ (30 μg litre^{-1}) fruits show higher values of PAL activity. In this way, the anthocyanin content and also its pattern, are consistent with this enzymatic activity (Fig. 2).

Nevertheless, in the plants which have received the higher concentration treatments of gibberellic acid ($GA_3(2)$), there is no clear correlation between the enzyme activity and the anthocyanin pattern (Fig. 2). In this case, the increase in anthocyanin content occurs before the maximum of PAL and TAL activities. This lack of correlation may be due to the biosynthesis of anthocyanins being limited by enzymes other than PAL/TAL, and/or differential effects on polyphenol metabolism at higher GA_3 concentrations.

CONCLUSIONS

Gibberellic acid (GA_3) treatments improve the weight and size of the strawberry fruits cv Chandler. Strawberries treated with GA_3 show higher anthocyanin content at 28 days than controls. Treatment $GA_3(1)$ (30 μg litre^{-1}) increased PAL activity which is correlated to the anthocyanin accumulation. Regarding TAL activity, this treatment also shows an increase of activity in all the days of the study except the 28 day, if compared to control. In contrast, $GA_3(2)$ (60 μg litre^{-1}) treatment does not show such correlation between enzyme activities and anthocyanin content, although this is the treatment that exhibits the highest anthocyanin content. Therefore, the results presented in this study establish that the responses obtained from different GA_3 treatments on strawberry plants depend on the dose applied.

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