BIOLOGIA PLANTARUM 41 (3): 475-479, 1998

BRIEF COMMUNICATION

The metabolism of inhibitor of flowering and prostaglandin biosynthesis, acetylsalicylic acid, in *Pharbitis nil* cotyledons

E.G. GROENEWALD and A.J. VAN DER WESTHUIZEN

Department of Botany and Genetics, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300, South Africa

Abstract

Acetylsalicylic acid, which applied to cotyledons of the short day plant *Pharbitis nil* prior to an inductive 16-h dark period inhibits flowering by 90 %, is converted to salicylic acid and to a lesser extent to gentisic acid in the cotyledons during this 16-h dark period. Our results confirmed that salicylic acid and gentisic acid are responsible for the inhibition of flowering. They also inhibit prostaglandin biosynthesis.

Additional key words: gentisic acid, inhibition of flowering, salicylic acid.

Prostaglandins (PGs) detected in many higher and lower plants (Lamacka and Sajbidor 1995, Groenewald and van der Westhuizen 1997), are thought to be involved in regulation of flowering of *Pharbitis nil*, a short day plant (SDP), since certain inhibitors (acetylsalicylic acid, phenylbutazone, indomethacin and niflumic acid) of PG-biosynthesis inhibit flowering (Groenewald and Visser 1974, 1978). Moreover, the application of certain PGs to excised apices grown in test tubes shortened the time to flowering (Groenewald and Visser 1974, 1978). Also, PGs have been shown to occur in both noninduced and induced to flower *P. nil* plants. The PG content of induced plants was 20 times higher than in noninduced (vegetative) plants (Groenewald *et al.* 1983). PGs may also be involved in the flowering regulation of another SDP, *Kalanchoe blossfeldiana,* since Janistyn (1982) could detect $PGF_{2\alpha}$ only in flowering plants and not in vegetative plants. PGs were also detected in the buds of *Populus balsamifera* (Levin *et al.* 1990). Recently Groenewald *et al.* (1994) found $PGF_{2\alpha}$ only in flowers and seed of *Oenothera stricta*. One of the potent

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Received 30 March 1998, *accepted* 10 August 1998.
Acknowledgements: This work was supported by the University of the OFS and the FRD.
Phone: (+51) 40 12260, fax: (+51) 44 88772, e-mail: vdwestaj@plk.nw.uovs.ac.za

inhibitors of PG-biosynthesis is *o*-acetylsalicylic acid and it was found that the application of this compound to cotyledons of *Pharbitis nil* prior to a 16-h dark period inhibited flowering of *P. nil* by 90 % (Groenewald and Visser 1974). The aim of this study was to determine the fate of acetylsalicylic acid during the 16-h dark period.

Pharbitis nil Chois. cv. Violet seeds were treated with concentrated H_2SO_4 (to soften the testa of the seed) and germinated as described by Groenewald and Visser (1974). On the 9th day after the germinated seeds were planted, a vaseline ring (10 mm in diameter) was made on each cotyledon of 40 seedlings and 1.85×10^4 Bq carboxyl-^{14}C labelled acetylsalicylic acid (0.01 cm^3) dissolved in 60 % ethanol was applied in the ring. At the same time, cotyledons of another 160 seedlings were dipped once in a 60 % ethanolic solution of unlabelled acetylsalicylic acid (2 g dm^{-3}). After 5 h the plants were placed in total darkness (27 °C) for 16 h after which the cotyledons were harvested. The vaseline ring was punched out (to get rid of unabsorbed labelled acetylsalicylic acid) and the rest of the cotyledons together with the cotyledons treated with unlabelled acetylsalicylic acid were used for determination of phenolic glycosides of low molecular mass according to Konishi and Galston (1964). The labelled and unlabelled cotyledons were pooled and homogenised in cold 50 % MeOH and left overnight in the dark in a cold room. The homogenate was filtered, evaporated under reduced pressure and the residue dissolved in 2 cm^3 distilled water. Amongst other compounds, the aqueous solution should contain glycosides of phenolic compounds, *e.g.*, salicylic acid and gentisic acid (Konishi and Galston 1964). The aqueous extract was chromatographed on *Whatman No. 1* paper (130 × 175 mm) sheets using *n*-butanol:acetic acid:H_2O (20:5:11 v/v) in the first direction and 5 % acetic acid in the second direction. Fluorescent spots (1 and 2) were detected under UV light. The chromatogram was then divided into 20 mm squares. The paper segments were placed in glass vials containing 1 cm^3 distilled H_2O and 9 cm^3 *Insta-gel* scintillator fluid and the radioactivity was counted in a *Tricarb* scintillation counter. In other experiments the fluorescent spots (1 and 2) were cut out and eluted with distilled water. The eluate was collected and hydrolysed according to Hestrin *et al.* (1955). The phenolic acids were extracted with diethyl ether and chromatographed on cellulose thin layer plates (20 × 20 cm; *Merck*, Darmstadt, Germany). For the first direction, the upper phase of a mixture of benzene:acetic acid:H_2O (6:7:3, v/v/v) and for the second direction a mixture of sodium formate:formic acid:H_2O (10:1:200, m/v/v) was used (Ibrahim and Towers 1960). The phenolic acids were identified under UV light and also after spraying with diazotized sulphanilic acid / Na_2CO_3 or diazotized *p*-nitroanaline / sodium acetate, which produced characteristic colours (red, yellow, orange and purple). In yet other experiments the cellulose plates, on which the phenolic acids were chromatographed, were divided into 20 mm squares and radioactivity of each square was determined as mentioned above. Spot 1 produced salicylic acid and gentisic acid and spot 2 only salicylic acid. The identity of the phenolic compounds was established by chromatography of the appropriate authentic compounds and by the colour reactions produced by the mentioned spraying reagents. Thirteen different

phenolic acids were chromatographed as standards which included gentisic acid, gallic acid and salicylic acid.

In other experiments the effects of gentisic, gallic and salicylic acids were tested on the flowering of *Pharbitis nil*. Plants were germinated and planted according to Groenewald and Visser (1974). Ten plants per treatment were used. Cotyledons were dipped once in the test solution [2 g dm^{-3}(acetylsalicylic acid in 60 % EtOH)]. Two sets of controls were also prepared. Firstly, the cotyledons of ten control plants were dipped only in 60 % EtOH. Secondly, ten plants received no treatment and no inductive cycle. After 5 h the plants received an inductive cycle of 16 h darkness (except the second set of controls). At the end of the dark period all the plants were subjected to long days (16 h light and 8 h darkness) for 29 d after which the control plants of the first set flowered.

According to the paper chromatographic analysis of the plant extracts (glycosides), most of the radioactivity was associated with the only two spots (1 and 2) which could be detected on the chromatogram in UV light. The amount of radioactivity in spot 1 was 44.72 counts per second (cps) and in spot 2 was 4.27 cps. Radioactivity recovered only from the spots that represented salicylic acid (2-hydroxybenzoic acid) was 31.52 cps and gentisic acid (2,5-dihydroxybenzoic acid) 4.65 cps in the case of the hydrolysate of spot 1. Percentages of radioactivity of salicylic and gentisic acids obtained from the hydrolysate of spot 1 were 87.14 and 12.86, respectively. The hydrolysate of spot 2 gave only one radioactive spot, namely salicylic acid (2.98 cps). Since no acetylsalicylic acid was detected on the chromatograms it is very likely that the acetyl group was hydrolysed in the cotyledons with the formation of salicylic acid. The salicylic acid was then converted to β-D-glucopyranosides in the cotyledons. This corroborates with the fact that low molecular mass phenolic compounds such as their β-D-glycosides naturally occur in plants (Harborne 1964). A small amount of gentisic acid was formed from the applied acetylsalicylic acid. The fact that the enzymatic hydrolysis of eluates from spots 1 and 2 each produced salicylic acid is an indication that at least two different β-glucosides of salicylic acid was produced from the applied acetylsalicylic acid.

According to Pridham (1965), *o*-acetylsalicylic acid does not occur in plants naturally, although both salicylic acid and gentisic acid do occur naturally in a variety of different plants. Ibrahim and Towers (1959) fed labelled salicylic acid to leaves of four different plant genera and found that in all cases it was converted to gentisic acid and o-pyrocatechuic acid (2,3-dihydroxybenzoic acid).

It is probable that the inhibition of flowering is not due to acetylsalicylic acid, since the acetyl group is hydrolysed by hydrolytic enzymes in the cotyledons, as mentioned earlier. The inhibition of flowering is then due to the salicylic acid and also to a certain extent by gentisic acid. They individually inhibited flowering of intact *P. nil* plants by 40 % and 70 %, respectively (Table 1). Taking into account the recovery from cotyledons the contribution to inhibition of flowering of salicylic acid to gentisic acid was 4:1. As already mentioned acetylsalicylic acid inhibited flowering by 90 % (Groenewald and Visser 1974). The greater effectiveness of acetylsalicylic acid relative to salicylic acid and gentisic acid in inhibiting flowering may be due to a better uptake of the former compound by the cotyledons of *P. nil*. It

should be kept in mind that acetylsalicylic acid, salicylic acid and gentisic acid are all inhibitors of prostaglandin biosynthesis (Flower 1974). It was found by Pryce (1972) that another phenolic acid, namely gallic acid (3,4,5-trihydroxybenzoic acid), is a natural inhibitor of flowering in the short day plant *Kalanchoe blossfeldiana*. It is not known whether gallic acid is an inhibitor of PG-biosynthesis, but it inhibited flowering of *P. nil* by 40 % (Table 1). In contrast, it was found that salicylic acid,

Table 1. The effect of gentisic, gallic acid and salicylic acids on flowering and shoot length (mean ± SD) of *Pharbitis nil*. Cotyledons were dipped once in compounds (2 mg cm^{-3}) dissolved in 60 % ethanol. Flowering was scored.

Compound tested in combination with a single inductive cycle	Number of plants that failed to flower	Shoot length [cm]
Gentisic acid	7	121 ± 6
Gallic acid	4	121 ± 5
Salicylic acid	4	118 ± 6
None (60 % ethanol)	0	89 ± 4
None and no inductive cycle	10	184 ± 9

which was excreted by aphids feeding on vegetative and reproductive shoots of the short day plant *Xanthium strumarium*, induced flowering in the long day plant *Lemna gibba* strain G_3 (Cleland and Ajami 1974). The stimulatory effects of salicylic acid on flowering of other species of *Lemna* were later demonstrated (Raskin 1992a). However, the possibility that salicylic acid functions as an endogenous regulator of flowering in *X. strumarium* and *Lemnaceae* is diminished by the fact that applied salicylic acid did not induce flowering in *X. strumarium*. Furthermore, no differences in the levels of salicylic acid in vegetative and flowering *X. strumarium* and *Lemna* could be detected. Moreover, the effect of salicylic acid on *Lemna* is not specific, since a large variety of benzoic acids, nonphenolic compounds including chelating agents, ferricyanide, nicotinic acid and cytokinins could induce flowering (Raskin 1992a). Recently salicylic acid has been implemented in signalling events leading to many plant responses (Raskin 1992b).

To summarise, the inhibition of flowering in *P. nil* was possibly caused by salicylic acid and gentisic acid, because applied acetylsalicylic acid was converted to the former two acids. Since both acids are known inhibitors of PG-biosynthesis these results confirm previous findings (Groenewald *et al.* 1983) that prostaglandins play a role in the flowering of *P. nil*.

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