Plant Physiol. (1974) 53, 644-648

# Levels of (+)-Abscisic Acid and Xanthoxin in Spinach under Different Environmental Conditions[1]

Received for publication September 21, 1973 and in revised form December 11, 1973

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## ABSTRACT

**The levels of the growth inhibitors (+)-abscisic acid and xanthoxin were determined in the long day plant spinach (*Spinacia oleracea* L. cv. Savoy Hybrid 612) grown under different environmental conditions. When plants were transferred from light to darkness, the (+)-abscisic acid level always decreased, whereas the xanthoxin content did not change. The (+)-abscisic acid content was higher in plants grown under low than under high relative humidity.**

**Xanthoxin levels were not affected by photoperiod, whereas the (+)-abscisic acid content increased 2 to 3 times upon transferring plants from short day to long day. Shoot tips with young leaves and mature leaves of the same plants analyzed separately did not differ in their inhibitor content when expressed per unit dry weight. No increase in xanthoxin level was observed in wilting plants. In general, the xanthoxin levels of spinach were much less affected by changes in the environment than were those of (+)-abscisic acid. In conclusion, there is no correlation between xanthoxin and (+)-abscisic acid levels in spinach on the one hand, and growth and flowering responses on the other.**

It was shown in earlier work that the LDP[2] spinach is more responsive to applied $GA_3$ with respect to stem growth under LD than under SD conditions (14). The possible interpretation of this phenomenon is that under SD growth inhibitors are nullifying the effect of GA. However, levels of the well known inhibitor, ABA, did not correlate with growth and flowering responses in spinach, indicating that ABA is not an endogenous regulator of these processes (12). Meanwhile, a neutral growth inhibitor, xanthoxin, with biological properties similar to those of ABA, has been discovered (9). Since xanthoxin has been detected in extracts of several higher plants (1), a study was made of possible changes in the xanthoxin levels when spinach plants are transferred from SD to LD where stem growth and flowering take place. Some additional experiments on variations in the ABA level under different environmental conditions are also reported.

[1] This work was supported by the United States Atomic Energy Commission under Contract AT(11-1)-1338.

[2] Abbreviations: LDP: long day plant; LD: long day(s); SD: short day(s); ORD: optical rotary dispersion; RH: relative humidity; *t*-ABA: 2-*trans*-abscisic acid; Me-ABA and Me-*t*-ABA: methyl esters of ABA and *t*-ABA; GLC: gas-liquid chromatography.

## MATERIALS AND METHODS

**Plant Material.** The growing conditions for spinach (*Spinacia oleracea* L. cv. Savoy Hybrid 612) were identical to those described previously (14). The flowering response and characteristics of petiole and stem growth of this variety have also been described (14).

In order to determine the water loss through transpiration from plants under different RH, the 340-ml plastic containers were placed in 400-ml beakers partially filled with water. This combination was weighed at the beginning and end of the high intensity light period. Corrections were made for evaporative losses from containers without plants.

Samples to be extracted consisted of 12 to 18 plants which after lyophilization ranged in dry weight from 12 to 28 g. All experiments were repeated at least once with similar results.

**Extraction and Purification Procedures.** Material which was analyzed for ABA only was extracted, and the acidic fraction was prepared and purified according to the procedures described previously (12). In addition, a method was developed which made it possible to determine the content of both inhibitors in one and the same sample. The various steps involved in this procedure are outlined in the flow sheet of Figure 1. Partitioning 80% aqueous methanol against petroleum ether effectively removed lipids. As established by Firn *et al.* (1), xanthoxin does not partition in the lipophilic phase until the proportion of methanol is lowered to 25%. For charcoal chromatography, 1 and 2 g of charcoal per 10 g dry weight were used for the acidic and neutral fractions, respectively.

**Thin Layer Chromatography.** Preparative TLC was carried out on 20 × 20 cm glass plates coated with Silica Gel $HF_{254}$ layers of 0.3 to 0.4 mm thick. Authentic spots of (±)-ABA and xanthoxin were co-chromatographed with the acidic and neutral fractions, respectively. The TLC systems used are listed in Table I. All elutions were done with water-saturated ethyl acetate. After purification of the neutral fraction in solvent system I, the eluted material was acetylated as described by Firn *et al.* (1), and re-chromatographed in system II.

The acidic fraction was purified successively in an acidic (III) and a basic (IV) solvent system. The eluted material was used for determining the (+)-ABA concentration by ORD as before (12). Some purified acidic samples were methylated with diazomethane prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis.). Methylated samples were re-chromatographed in solvent system V before being analyzed by GLC.

**Gas-Liquid Chromatography.** All the results reported were obtained with a Hewlett-Packard Model 402B gas chromatograph equipped with dual flame ionization detectors and U-shaped glass columns (1.83 × 3.0 or 3.5 mm).

For analysis of the neutral fraction containing O-acetylxanthoxin the stationary phase OV-17 was used. The column

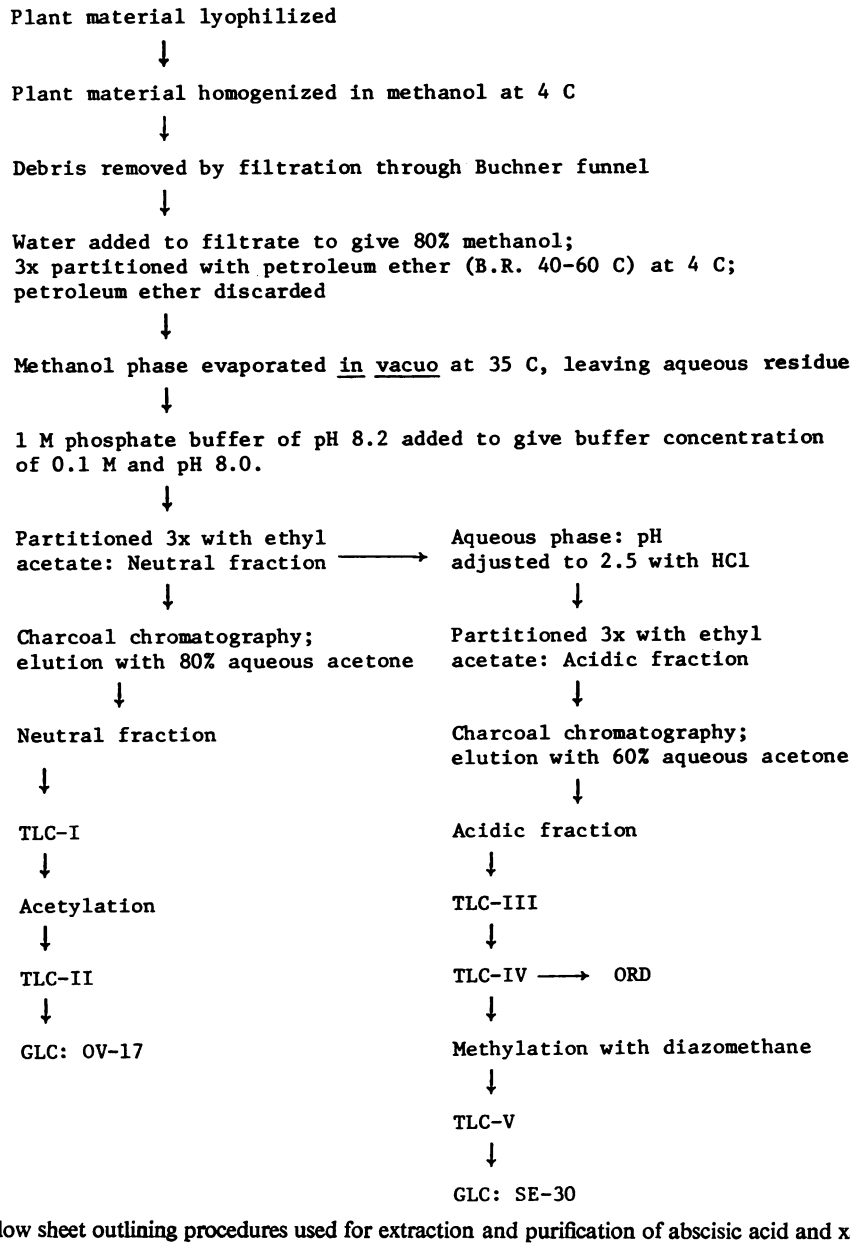


FIG. 1. Flow sheet outlining procedures used for extraction and purification of abscisic acid and xanthoxin.

Table I. *Thin Layer Chromatography Solvent Systems used to Purify Xanthoxin and ABA*

Plates (20 × 20 cm) were coated (about 400 μm thick) with Silica Gel $HF_{254}$. Plates were developed to 15 cm, except solvent system IV which was run only 12 cm.

| Solvent Systems | $R_F$ Values |
|---|---|
| | Xanthoxin |
| I. Hexane-ethyl acetate (2:3) | 0.27–0.40 |
| | O-acetyl xanthoxin |
| II. Hexane-ethyl acetate (3:2) | 0.60–0.73 |
| | ABA |
| III. Chloroform-ethyl acetate-acetic acid (60:40:5) | 0.52–0.60 |
| IV. 1-Butanol-1-propanol-ammonia-water (2:6:1:2) | 0.54–0.62 |
| | Me-ABA |
| V. Hexane-ethyl acetate (1:1) | 0.65–0.71 |

packing was prepared by filter-coating a 1.5% (w/v) solution in toluene-chloroform (1:1, v/v) on Gas-Chrom Q 80/100 mesh. The samples were analyzed isothermally at 210 C with both the injection port and detector at 240 C. The flow rate of the carrier gas, $N_2$, was 40 ml/min. The two isomers of xanthoxin were identified by comparison with an authentic sample of O-acetyl-xanthoxin, containing approximately equal amounts of the two isomers. For quantitative determinations the peak areas were measured by multiplying peak height with half peak width. Over the working range used, a linear relationship was established between the amount of xanthoxin injected on the column and peak area.

For analysis of the methylated acidic fraction containing Me-ABA, a column with SE-30 as stationary phase was chosen. The packing was prepared by filter-coating a 3% (w/v) solution in chloroform on Gas-Chrom Q 80/100 mesh. Conditions used for separation of Me-ABA and Me-*t*-ABA were: oven temperature 175 C, injection port 220 C, and detector at 240 C; flow rate of the carrier gas, $N_2$, at 55 ml/min.

## RESULTS

**Effect of Light and Darkness on ABA Levels.** As shown in Figure 2, the ABA content of spinach plants grown under SD conditions fluctuates, rising during the 8-hr light period and decreasing in darkness. Although the observed differences were not large, they could be reproduced consistently. Lenton *et al.* (2) also observed a diurnal variation in the ABA content of the apical organs from birch seedlings grown under SD, except that the lowest levels were found at the end of the high intensity light period.

When plants were exposed to LD and then transferred to darkness for 16 hr, the ABA level dropped to that of plants in SD, whereas under weak supplementary light the level remained constant (Fig. 3). The results of a similar experiment are given in Table II, except that the amount of ABA released by alkaline hydrolysis was also determined. After 8 hr of darkness the level of free ABA had decreased about 50% with a concomitant increase in a hydrolyzable metabolite, presumably the glucose ester of ABA. An increase in the level of this compound was also found when plants recovered from wilting (10).

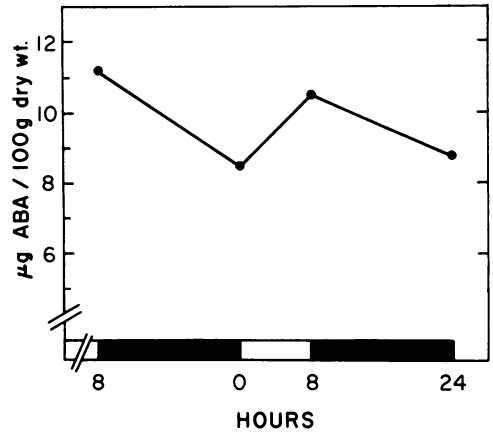


FIG. 2. Fluctuation of abscisic acid level in spinach plants grown under short days.

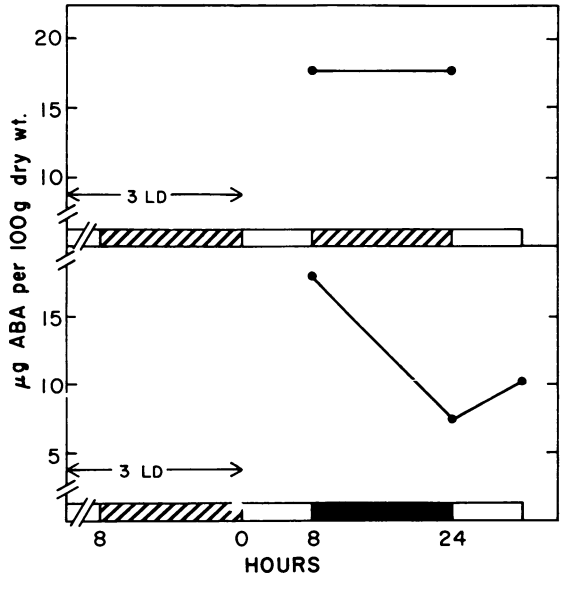


FIG. 3. Abscisic acid level in spinach under long-day conditions, and its decline after transfer to darkness. Open bars: high intensity light; hatched bars: weak supplementary light from incandescent lamp; solid bars: darkness. Temperature was 23 C throughout.

Table II. *Effects of Light and Darkness on the Level of ABA in Spinach*

Plants were exposed to high intensity illumination from 8 AM until 4 PM; weak supplementary irradiation from incandescent lamps was given at an intensity of 40 ft-c for the remaining 16 hr. RH: during light 35%, in darkness 80%.

| Treatment | Time of Harvest | ABA Content | |
|---|---|---|---|
| | | Free | After hydrolysis[1] |
| | | *µg/100 g dry wt* | |
| 4 LD | 4 PM | 18.6 | 4.4 |
| 4 LD, 16 hr supplementary light | 8 AM | 19.6 | 3.3 |
| 4 LD, 8 hr darkness | Midnight | 10.5 | 7.8 |
| 4 LD, 16 hr darkness | 8 AM | 7.5 | 2.0 |

[1] Conditions: pH 11.0, 60 C, 30 min (12).

Table III. *Xanthoxin Content of Spinach after Exposure to Increasing Numbers of Long Days*

Plants were harvested at end of 8-hr high intensity light period.

| Photoperiodic Treatment | Xanthoxin Content | |
|---|---|---|
| | *cis,trans* | *trans,trans* |
| | *µg/100 g dry wt* | |
| 0 LD | 27.7 | 76.0 |
| 4 LD | 27.6 | 79.0 |
| 8 LD | 25.2 | 73.4 |

**Effect of RH on ABA Levels.** When spinach plants were grown under LD and a RH of 35 or 75%, the ABA content at the end of the 8-hr high intensity light period was 13.2 and 8.9 µg/100 g dry weight, respectively. During the 8-hr light period, plants in the low RH lost approximately twice as much water through transpiration as did those under high RH (79 *versus* 34 g water lost per plant). Despite the difference in ABA content under the two conditions, stem growth was not significantly different: 49 cm at 35% RH and 48 cm at 75% after 24 LD. This indicates that the higher ABA level associated with low RH did not inhibit stem growth. Working with peas, Simpson and Saunders (7) also discounted the possibility that ABA controls internode growth of peas.

**Xanthoxin Levels and Photoperiod.** The results in Table III show that the xanthoxin level did not change appreciably when plants were transferred from SD to LD. *Cis,trans*-xanthoxin was always accompanied by larger amounts of the *trans, trans*-isomer (Table III, Fig. 4).

The endogenous GA level of spinach shoot tips with immature leaves is higher than that of the older leaves (13). These two types of organs were therefore also analyzed separately for their growth inhibitor content (Table IV). The xanthoxin level was remarkably constant in all tissues under both SD and LD. The same was true for the ABA content, except that there was a 2-fold increase under LD as compared to SD. In birch, on the other hand, apices and half-expanded leaves contained two to three times more ABA on a fresh weight basis than mature leaves (2).

**Effect of Darkness on Xanthoxin Levels.** Xanthoxin can be

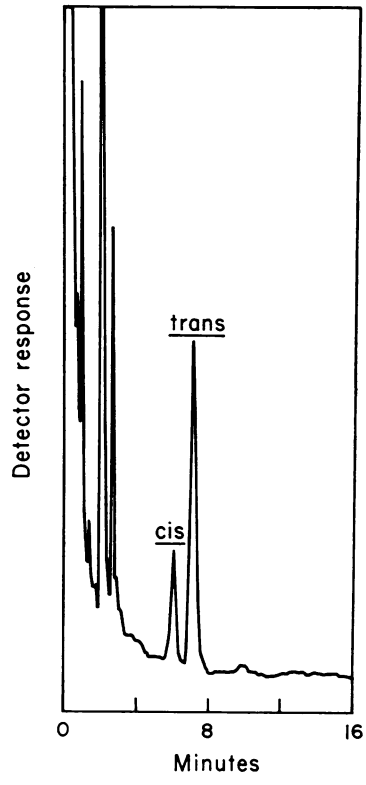


FIG. 4. Gas chromatogram of neutral fraction containing *cis,trans*-O-acetyl xanthoxin and *trans,trans*-O-acetyl zanthoxin prepared from spinach plants exposed to 8 long days. The 1 μl of extract injected was equivalent to 0.2 g of dry weight. Range was 10, attenuation was 16×.

Table IV. *Levels of ABA and Xanthoxin in Spinach Shoot Tips and Leaves grown under SD or after 8 LD*

Tips: shoot tips + immature leaves; leaves: mature leaves; total: data of tips and leaves combined.

| Photoperiodic Treatment | Plant Parts | ABA Content | *Xanthoxin Content* | |
|---|---|---|---|---|
| | | | *cis,trans* | *trans,trans* |
| | | *μg/100 g dry wt* | *μg/100 g dry wt* | |
| SD | Tips | 8.8 | 21.8 | 56.5 |
| | Leaves | 8.7 | 25.9 | 71.6 |
| | Total | 8.8 | 24.5 | 66.5 |
| 8 LD | Tips | 15.2 | 23.7 | 65.6 |
| | Leaves | 14.9 | 24.5 | 72.0 |
| | Total | 15.0 | 24.2 | 69.4 |

prepared *in vitro* by photooxidation of xanthophylls (9). It was therefore of interest to compare the xanthoxin content of plants after 8-hr high intensity light and at the end of a 16-hr dark period. The results in Table V show that the xanthoxin content remained the same in light and darkness, while the ABA level had decreased as before. In subsequent experiments, the dark period was extended further, but even after 31 hr of darkness the *cis,trans*-xanthoxin content had decreased by only 15%.

**Effect of Wilting on ABA and Xanthoxin Levels.** In wilting spinach, the ABA level increased 10 times over that of turgid plants (12). An experiment was carried out therefore to see if the xanthoxin content is also affected by wilting. As shown in Table VI, a 4-hr wilting period increased the ABA level as before (12). However, there was no corresponding rise in the xanthoxin content of wilted plants. Only in plants under SD was there a slight increase in xanthoxin. Part of the ABA samples extracted from wilted plants were also analyzed by GLC. As shown in Figure 5, the Me-ABA was accompanied by a small amount of the biologically inactive Me-*t*-ABA. It is possible that this was the result of isomerization when viewing the thin layer plates under UV.

## DISCUSSION

It was concluded earlier (12) that ABA does not function as an endogenous inhibitor of flower formation and stem growth of spinach in SD. On the basis of the results described above, the same conclusion applies also to xanthoxin, since no differences in the level of this inhibitor were found in spinach plants grown under SD and LD (Tables III and IV). On the other hand, the higher growth rate of petioles under LD (14) is associated with an increase of the endogenous GA level in the shoot tips, particularly of $GA_{20}$ (13). Present evidence favors the idea that LD-induced growth responses in spinach are mediated by changes in the endogenous GAs.

The level of ABA in leaves can be raised by a variety of conditions: wilting (10, 11), waterlogging of the root system (10), low RH (4, 10), osmotic stress of the roots (5), lack of mineral nutrients (6), cold stress (3), and infection with the wilt-inducing bacterium *Pseudomonas solanacearum* (8). A common feature of these factors is that they affect the water balance of plants. Apparently, whenever a water deficit is generated in leaves, the adaptive response of plants is to increase their ABA level which results in stomatal closure, thus reducing further water loss (10). In the present experiments with spinach, LD, low RH, and wilting all raised the ABA level while leaving the xanthoxin level unchanged. Thus, xanthoxin levels are much less subject to fluctuation under different en-

Table V. *ABA and Xanthoxin Content of Spinach Plants as Affected by Light and Darkness*

Light conditions as in Table II. RH: in light 40%, in darkness 55%.

| Treatment | Time of Harvest | ABA Content | Xanthoxin Content | |
|---|---|---|---|---|
| | | | *cis,trans* | *trans,trans* |
| | | *μg/100 g dry wt* | *μg/100 g dry wt* | |
| 5 LD | 4 PM | 11.9 | 20.1 | 57.5 |
| 5 LD, 16 hr supplementary light | 8 AM | 11.5 | 24.1 | 66.9 |
| 5 LD, 16 hr darkness | 8 AM | 6.5 | 21.0 | 64.9 |

Table VI. *ABA and Xanthoxin Content of Spinach as Affected by Photoperiod and Wilting*

| Treatment | ABA Content | Xantoxin Content | |
|---|---|---|---|
| | | *cis,trans* | *trans,trans* |
| | *μg/100 g dry wt* | *μg/100 g dry wt* | |
| SD | 7.1 | 18.4 | 39.3 |
| SD, wilted[1] | 101.1 | 27.7 | 74.3 |
| 4 LD | 14.7 | 24.8 | 73.9 |
| 4 LD, wilted[1] | 120.8 | 24.1 | 62.5 |

[1] Fresh weight of plants without roots reduced by 10% and kept at 22 C for 4 hr.

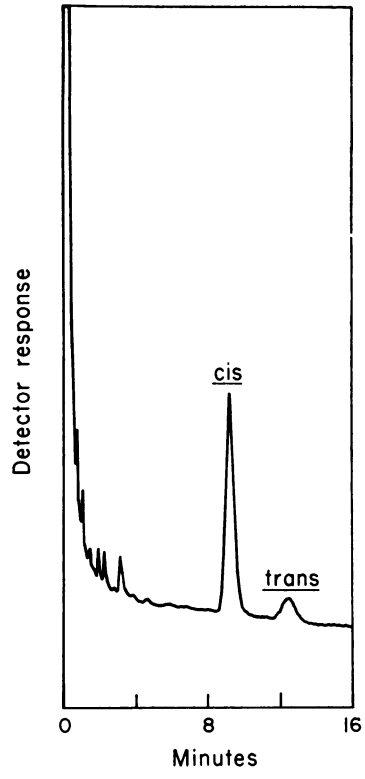


FIG. 5. Gas chromatogram of acidic fraction containing Me-ABA and a trace of Me-*t*-ABA prepared from wilted spinach plants after 4 LD (Table VI). The 1 $\mu$l of extract injected was equivalent to 0.4 g of dry weight. Range was 10, attenuation was 16×.

vironmental conditions than are those of ABA. While the regulatory role of ABA in stomatal closure is now well known (10, 11), the physiological role of xanthoxin remains to be established.

The leaves of spinach plants grown under LD are thinner and of a lighter green color than those of plants in SD. When detached, the former wilt more rapidly. It is possible therefore that the increase in ABA content after transfer from SD to LD (Tables IV and VI) is not a direct photoperiodic effect, but rather an expression of the water status of the leaves, which may be approaching incipient wilting.

*Acknowledgments*—I am much indebted to Dr. R. D. Firn for advice with analysis of xanthoxin and for use of an authentic sample of xanthoxin. Gifts of synthetic (±)-ABA were kindly supplied by the Reynolds Tobacco Company and by Hoffmann-LaRoche. I am indebted to Tiit Kivilaan for technical assistance with extractions.

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