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Stress induced ethylene evolution and its possible relationship to auxin-transport, cytokinin levels, and flower bud induction in shoots of apple seedlings and bearing apple trees

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Abstract

Mechanically induced stress (MIS) was imposed on apple shoots by bending horizontal shoots upward and vertical shoots downward and its effect on internal ethylene evolution, polar auxin transport and cytokinin levels studied. Induction of stress caused a significant rise in internal ethylene production in the tissues under stress, decreased polar auxin transport and cytokinin levels and increased the percentage of floral buds in the shoots. There were only quantitative differences between bending up or down of the shoots. In general, unstressed horizontal shoots had higher ethylene production rates and exhibited lower auxin transport and cytokinin levels compared to vertical shoots which may explain the reason for higher flower bud formation. Parallel trials with seedlings using the ethylene action inhibitor silver-thiosulphate (STS) confirmed that ethylene concentrations in the tissues may be responsible for the reduction of endogenous basipetal auxin transport and Z/ZR levels in the shoots. It is suggested that the main stimulae for mechanically induced stress responsiveness in shoots of apple plants is ethylene and ethylene regulated changes in auxin transport and cytokinin levels. Flower bud formation may be regulated by some of these phytohormonal changes.

Abbreviations: ACC = 1-aminocyclopropanecarboxylic acid; C_2H_4 = ethylene; IAA = indoleacetic acid; iAde/iAdo = isopentenyladenine/isopentenyladenosine; Z/ZR = zeatin/zeatinriboside; MIS = mechanically induced stress; STS = silver-thiosulphate

1. Introduction

Occurrence of natural mechanically induced stress (MIS) has a wide range of influences on growth and development of plants and the responses to MIS also varies from species to species [9]. Increases in ethylene evolution following MIS has been reported by several workers [19, 21, 26, 27] and may be important in controlling the MIS induced growth responses [9].

Bending or horizontal placement of vigorously growing shoots often reduces growth and stimulates flowering in apple [33, 35] possibly because of changed patterns of hormone transport [1], distribution, or a changed pattern of sensitivity to hormones [29]. Ethylene is an important regulator of flower bud formation particularly in pineapple but also in apple [20, 32]. Ethylene has been reported to reduce endogenous IAA levels and transport in plants [13, 22, 25] and some of the ethylene regulated physiological roles in plants have been suggested to be mediated through this effect [13, 22, 39]. In addition an antagonism has recently been noted between ethylene and cytokinins [36]. MIS stimulated flower bud induction could thus be mediated by elevated ethylene production, which could alter IAA transport and cytokinin concentrations.

In the present study we examined MIS (by shoot bending) induced ethylene production and its influences on auxin transport and cytokinin levels in relation to flower bud formation of apple shoots.

2. Materials and methods

2.1 *Experiment I*

2.1.1 *Plant materials*

The experiments were carried out at the Research Station of the University of Hohenheim with 18-year-old apple trees (*Malus domestica* Borkh.) cv. Boskoop. Vigorously growing horizontal and vertical shoots (300 each) were seleceted for this study. Half of the vertical shoots were bent down and half of the horizontal shoots were bent up and held in position with rubber bands in the middle of June. The experiment consisted of four treatments, namely: i) horizontal, ii) vertical, iii) vertical bent down, and iv) horizontal bent up of shoots. Sampling was done (20 shoots treatment^{-1} day^{-1}) 1, 2, 4 and 7 days after bending. No material was collected from horizontally bent up shoots on the 7th day as almost all these shoots had stopped growing after this time. Sampling from horizontal and vertical control shoots were performed together with treated shoots.

2.1.2 *Diffusible IAA*

The shoot tips were excised with a sharp razor blade. All the three-quarter to fully developed leaves were removed and the cut ends immediately immersed into multititer plates containing 2 ml of phosphate-buffer (0.1 M, pH 6.2) per well. Two shoot tips were put in each well. The plates were then incubated in the dark at 20±2 °C and almost 100% RH for 20 h. The shoot tips were then removed and the plates stored at −20 °C untill further analysis.

For analysis of IAA the diffusates were thawed, purified by adjusting the pH to 8.5 and then passing them through first a PVP column and then, after adjusting the pH to 3.0, over C-18 Sep-Pak cartridges (Waters, Milford, USA). The cartridge was then washed first with 4 ml of 0.1 M acetic acid followed by 4 ml methanol 25% (v/v) in 0.1 M acetic acid. IAA was finally eluted with 4 ml methanol 40% (v/v) in 0.1 M acetic acid and quantified, after evaporating the solvent, by radio-immunoassay following a method described by Bohner and Bangerth [11]. All immunoassays were performed in triplicate. The losses of IAA during purification and determination were estimated by adding 1-[^{14}C]-IAA (Amersham, spec. activity 1.05 GBq m mol^{-1}) as an internal standard and the final results were corrected accordingly.

2.1.3 *Cytokinin analysis*

Four cm of the subapical part of each shoot was collected just after the shoot tips had been sampled for auxin transport measurement. The cut portions were immediately frozen in liquid nitrogen, lyophilized, homogenized and stored in a refrigerator at −20 °C. For analysis, samples (250 mg) were extracted overnight with methanol 80% (v/v) at 4 °C. The samples were then filtered, evaporated to dryness, and then taken up into P-buffer (0.1 M, pH 8.5) in centrifuge tubes and frozen over night. The frozen samples were thawed, centrifuged at 40.000 g and the supernatant purified passing it first through a PVP column and, after adjusting the pH to 3.0, over C-18 Sep-Pak cartridges. The cartridge was then washed with 4 ml 0.1 M acetic acid and Z/ZR was eluted with 4 ml methanol 25% (v/v) in 0.1 M acetic acid; iAdo/iAde was finally eluted with 4 ml methanol 70% (v/v) in 0.1 M acetic acid. The solvent was evaporated and CKs were quantified by radioimmunoassay using polyclonal antibodies [11]. All immunoassays were performed in triplicate.

2.1.4 *Ethylene estimation*

The bended portions of the shoots (10 cm long) were severed and immediately put into 50 ml syringes after removal of the leaves, and the syringe was closed with a rubber septum. Ten severed parts from each treatment were put in one syringe. The representative parts of the unstressed horizontal and vertical shoots were sampled in the same way. The syringes were then incubated at 20±2 °C for 3 h and ethylene was measured using a gas chromatograph fitted with an activated alumina column and a FID detector.

2.1.5 *Estimating potential flower buds*

The potential flower buds of the shoots were estimated following the method described by Skene [31] with little modifications. Shoots (35–45 cm) were severed during February just below the part under stress and inserted in trays containing moist vermiculite and kept in the glasshouse at a temperature of 16 °C for one week and thereafter at 20 °C. Observations were taken after 4 weeks by dissecting the swelled buds under a binocular microscope. Buds that did not develop

at all were not considered for observation. Data are presented in terms of percentage.

2.2 *Experiment 2*

2.2.1 *Plant materials and treatment allocation*

To confirm the relationship between internal ethylene accumulation and auxin transport and CK levels (see below) another experiment was carried out in the glasshouse with apple seedlings raised from stratified seeds of the cv. Golden Delicious in a partially controlled atmosphere (temperature 25/18 °C day/night). Uniformly germinated seedlings were grown in 11 cm pots (one seedling/pot) filled with a peat-soil mixture. Seedlings were watered regularly and fertilized at 15 day interval with a complete liquid fertilizer (Wuxal top N, Aglukon, Germany). They were occassionally sprayed with Saprol Neu and Metasystox (Shell Agrar, Ingelheim, and Bayer, Leverkusen, Germany respectively) to prevent attacks of powdery mildew and aphids. This experiment was conducted during winter time and the natural light was supplemented with 250 μmole, provided by 400 W HQI lamps (Osram, Germany) for 4 h daily in the evening from the end of October onwards. Seedlings were 5 months old during the period of study. Three treatments were used namely: i) control, ii) bending down, and iii) bending down + silver-thiosulphate (STS) (4 mM). Each treatment consisted of 15 plants. Silver-thiosulphate was sprayed over the plants one day before bending. Seedlings were tender and so they were bent down by fine cotton threads. Control plants were left untouched.

2.2.2 *Determination of ethylene, auxin diffusion and cytokinins*

Estimation of different hormones were done only once i.e. 48 h after bending. The methods followed were the same as described above.The exceptions were, only one shoot tip was immersed in each well containing phosphate-buffer and ethylene evolution in the stressed part was measured separately in leaf and stem tissues.

The results of both the experiments were analysed by ANOVA and the means were separated by Duncan's multiple range test.

3. Results

3.1 *Ethylene evolution*

Both the bending treatments considerably increased ethylene production of the bearing trees in the plant parts under stress (Figure 1). Bending down had a stronger effect than bending up treatments, throughout the period of investigation. In general, the tissues of untreated horizontal shoots produced more ethylene than vertical shoots. Daily variations in ethylene production were probably due to changes in environmental conditions, notably temperature.

Similarly, bending down as well as STS + bending down treatments with seedlings caused marked increases in internal C_2H_4 production irrespective of whether leaf or stem tissues were measured (Table 1). This confirmed the results with mature trees. Estimation of ethylene evolution of the shoot tips collected from bent shoots failed to give any significant variation when compared with control even after 18 h of incubation (data not shown).

3.2 *Auxin transport*

Mechanically induced stress applied to vigorously growing vertical shoots caused a 2 to 2.5 fold decrease in polar auxin transport when compared to control vertical shoots (Figure 2). In general, the horizontal shoots exhibited considerably less IAA transport when compared to vertical shoots, which further tended to decline, though not significantly, when the shoots were put under stress. Similar trends of responsiveness to MIS in respect to IAA transport was observed in the seedling trials (Table 1). Higher auxin transport in the seedling plants treated with STS + bending clearly suggest that the reduction in auxin transport in response to MIS is at least partially an effect of the rise in ethylene concentration in the tissue, although shoot tips of bended shoots did not show elevated ethylene production (see above). However, a critical analysis of the data obtained in control horizontal and horizontal shoots bent up showed that ethylene inhibition of auxin transport was limited. A quick cessation of growth in horizontal shoots bent up was observed and was possibly due to decreased auxin transport and high ethylene accumulation following MIS.

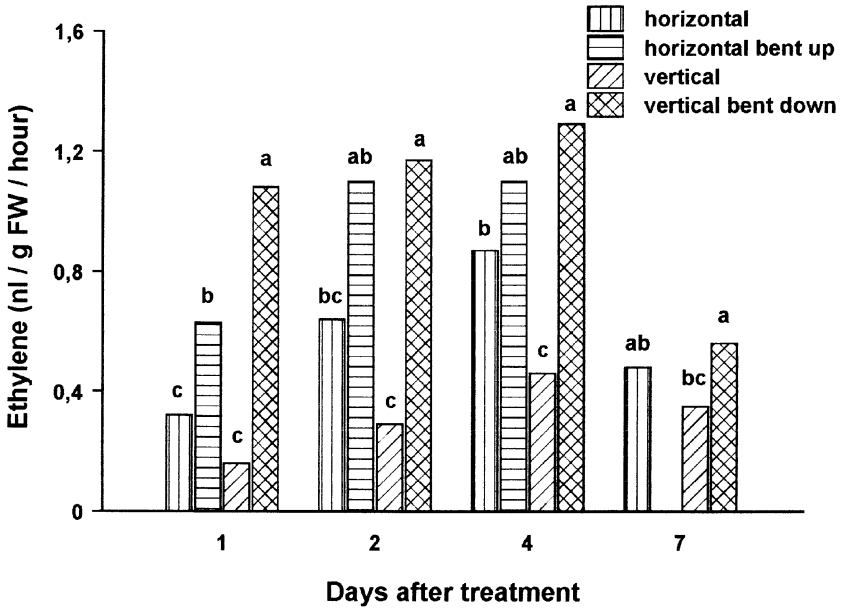

Figure 1. Effect of shoot orientation and mechanically induced stress on ethylene production of shoots of mature apple trees.

Table 1. Effect of stress and STS application on ethylen evolution, auxin transport and cytokinin levels of apple seedling

Treatment	Ethylene ($n1 \cdot g^{-1} \cdot h^{-1}$)	IAA-transport ($ng \cdot$ IAA $apex^{-1}$)	Z/ZR concentration ($ng \cdot g^{-1}$ D.W.)	iAde/iAdo concentration ($ng \cdot g^{-1}$ D.W.)
Control	0.6	8.9 a	21.6 a	20.5 a
Bending down	5.2	6.1 b	15.0 b	28.6 a
Bending down + STS spray	6.7	8.9 a	22.6 a	23.3 a

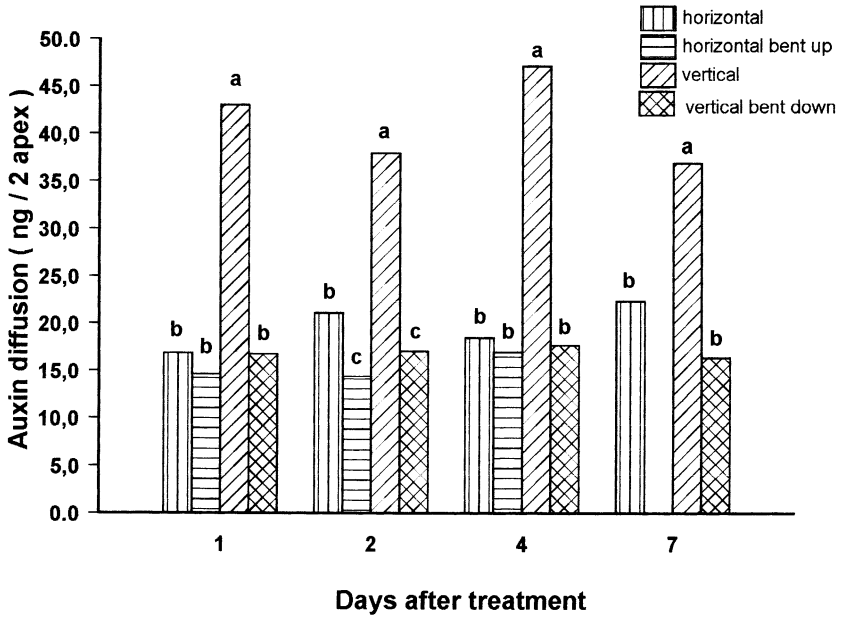

Figure 2. Stress induced changes in auxin (IAA) diffusion of shoot tips of mature apple trees.

Table 2. Stress induced variation in percentage of floral and vegetative buds in apple shoots after forcing

Treatments	% Terminal buds		% Lateral buds	
	F	V	F	V
Horizontal	68.8	31.2	38.6	61.4
Vertical	–	–	14.6	85.4
Vertical bent down	75.0	25.0	38.9	61.1
Horizontal bent down	85.0	15.0	39.4	60.6

F = floral buds V = vegetative buds

3.3 *Z/ZR levels*

The Z/ZR levels in apple shoots were also found to be highly sensitive to MIS (Figure 3). Displacement of vertical shoot downward caused a nearly 3-fold decrease in Z/ZR content. In general, horizontal shoots had low Z/ZR levels which further declined when shoots were subjected to stress. Experiments with apple seedlings confirmed these results (Table 1) suggesting a possible influence of ethylene also on Z/ZR levels in plants because STS alleviated the MIS induced decline in Z/ZR concentration.

3.4 *iAdo/iAde levels*

No significant variation, in iAdo/iAde levels in the tissues could be found as a result of MIS (Figure 4). In general, reorientation of vertical shoots by bending down resulted into reduced levels of iAdo/iAde, while displacement of the horizontal shoot upward had practically no effect. Results with seedlings were similar (Table 1).

3.5 *Percentage of floral buds*

Both the bending treatments increased the percentage of floral buds in shoots, irrespective whether terminal or lateral positions were compared to the controls (Table 2). In general, the horizontal shoots had more floral buds than vertical shoots. None of the apical buds of the vertical shoots developed during the period of forcing. The number of shoots and buds was too small to perform a statistical analysis of the results.

4. Discussion

Induction of MIS caused a several fold increase in ethylene evolution in the tissues which is in agreement with findings of several workers [10 26, 27, 31]. In addition ethylene production was higher in all the sections of horizontal apple shoots than in corresponding parts of vertical shoots. This suggests an effect of gravitational forces beside MIS on ethylene production and, hence, on gravimorphic events in apple trees. Variation in the levels of ethylene production between juvenile (seedlings) and adult tissues in the present study may be due to their variation in responsiveness to stress as well as to the different environments. Induction of mechanical stress in several plants causes instantaneous rise in callose, a cell wall polysaccharide, in the section under stress, which may led to the activation of rate limiting ACC-oxidase that catalyzes the conversion of ACC to ethylene [18]. This is an alternative explanation for the observed rise in ethylene production of MIS-treated shoots than the implication of gravitational forces, because it is observed in those shoots bent up as well.

Polar auxin transport in MIS treated shoots was reduced which is, at least partially, thought to be due to increased ethylene production in the tissues under stress. Comparisons between normal horizontal and vertical shoots also indicated the negative role of C_2H_4 on IAA transport. This was further confirmed by the STS + bending treatment, where, despite high ethylene production, IAA transport remained the same as observed in controls. Silver (Ag^+), is a potent inhibitor of ethylene action in plants [7, 37, 38].

Several workers have reported the inhibition of auxin synthesis and transport by ethylene in plants [6, 22, 24]. Ethylene inhibition of polar IAA transport may be ascribed to reduced continuous synthesis [13], less transport from the site of synthesis [6] and/or increased conjugation and decarboxylation during transport [8, 25, 28].

The reduction in the levels of Z/ZR in the MIS treated tissues, irrespective whether adult or juvenile material was analysed, may be either a direct or indirect effect of high ethylene accumulation in those shoots. The exact relationship between endogenous ethylene and CKs is not yet clear. However, increased ethylene may decrease the levels of CKs in plants [4, 36]. In addition, the fact that STS-treatment alleviated the effect of bending on the concentration of Z/ZR again suggests that reduction in Z/ZR in bent tissue was the result of stimulated ethylene production. Therefore, increased ethylene production might be the primary event after MIS and reduced IAA transport and cytokinin concentration consequences of the elevated ethylene production. This observed relation-

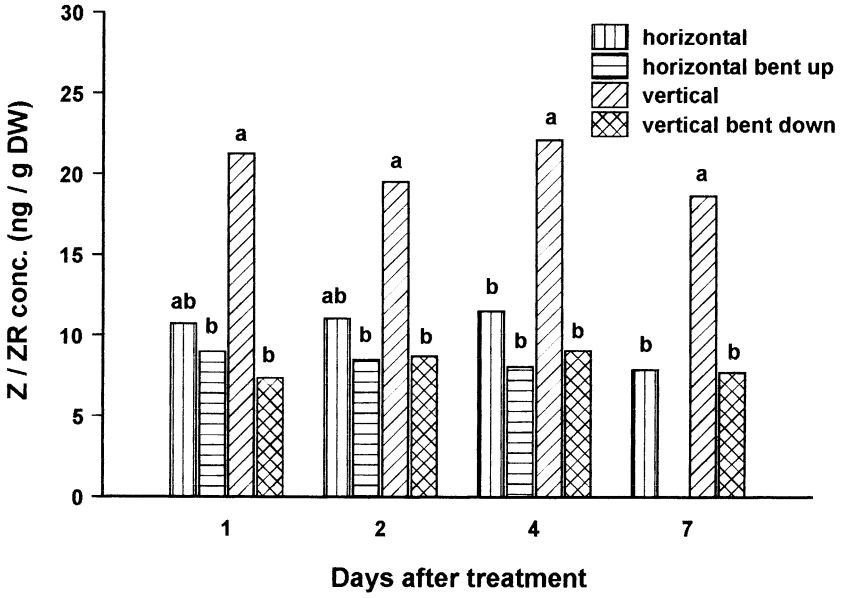

Figure 3. Stress induced changes in the endogenous concentration of the cytokinins zeatin/zeatinriboside (Z/ZR) in shoots of mature apple trees.

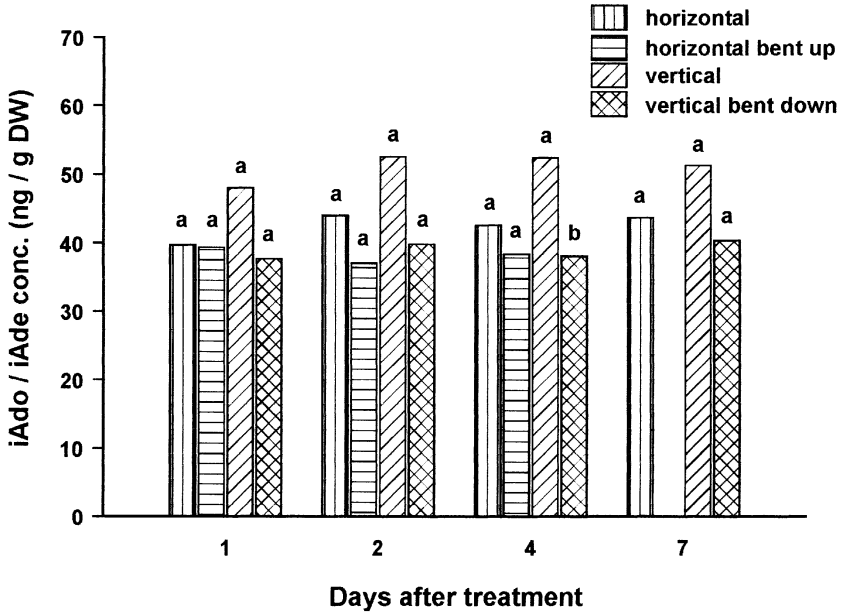

Figure 4. Effect of stress on the endogenous concentration of the cytokinins isopentenyladenine/isopentenyladenosine (iAde/iAdo) in shoots of mature apple trees.

ship between IAA transport and and CK concentration is thus opposite to what is found in annual plants [2].

The main purpose of these investigations was to elaborate the possible involvement of endogenous hormones in flower bud formation. Bending of shoots seems to be a good tool to achieve this goal. It is well known that this procedure stimulates flower bud induction [33, 35] and at the same time increase ethylene formation [26, 27]. Together with experiments which demonstrated a positive effect of Ethrel applications on flower bud formation [20, 32] it seems, therefore, likely that bending of shoots mediates its effect on

flower buds via an altered ethylene production. An alternative explanation would be the involvement of polar IAA transport in the morphogenetic event of flower bud initiation. It was suggested that IAA transport could be an inhibiting signal in this event [1, 15] and its reduction could, therefore, stimulate flower bud induction. The effect of bending could thus express itself by an initial increase in ethylene production, which then reduces polar IAA transport which finally stimulates flower bud formation. The involvement of IAA transport is substanciated by "ringing experiments", which showed a decrease in IAA transport in the shoot (Gruber and Bangerth, in prep.) and stimulated flower bud formation [12, 34]. Even more conclusive in this respect are the experiments by Ben-Tal and Lavee [5] who demonstrated an increase in flower buds in olive trees after "chemical ringing" with the auxin transport inhibitor chlorofluorenol.

In contrast to ethylene and IAA the involvement of endogenous cytokinins in flower bud formation is presently unclear. Whereas the exogenous application of these hormones seems to stimulate flower buds [14, 17, 23], a relationship with the endogenous concentration of these hormones is, however, far less clear [34]. The above results do not contribute much to clarify this contradiction. Bending decreased cytokinin concentration as does girdling above the girdle [12, 34]. As to whether this indicates that flower bud formation requires low endogenous cytokinin concentration, however, is uncertain. Analysis of conjugated cytokinins and at the side of flower bud formation, which means in the buds themselves, would possibly be a better approach to clarify cytokinin: flower bud interactions than analysing whole shoot material.

It is evident from the above results that one of the main stimuli for MIS responsiveness to plants is ethylene and ethylene regulated alterations in other endogenous hormonal balances responsible for the expression of MIS induced morphogenetic responses, like flower bud induction.Bending seems to be a good tool to further elaborate the involvement of plant hormones in the flower induction process.

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