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Water stress and root formation in pea cuttings

III. Changes in the endogenous level of abscisic acid and ethylene production in the stock plants under two levels of irradiance

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By

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Abstract

Eleven day old pea plants (*Pisum sativum* L. cv. Alaska), grown at 16 W m^{-2} or 38 W m^{-2} were stressed with different concentrations of polyethyleneglycol (PEG): –3.0 (mild), –6.4 (moderate) or –13.9 bar (severe) for 2, 4 or 6 h. The leaf osmotic potential, ψ_π, and the endogenous abscisic acid (ABA) level and ethylene production were determined at the end of the stress treatment.

The ψ_π decreased with stress treatments. The reduction was greater at 38 W m^{-2} than at 16 W m^{-2}; severely-stressed plants had less ABA content than those under moderate or mild stress. Ethylene production was higher with the 2 h stress treatment than the non-stressed only at 38 W m^{-2}. Prolongation of stress to 6 h decreased the ABA level and also ethylene production under both levels of irradiance.

The relationship between the changes in ABA and ethylene and the adventitious root formation in pea cuttings is discussed.

Key-words: Water stress; ABA; Ethylene; Irradiance; Pea; Polyethyleneglycol; Leaf osmotic potential; Adventitious root formation.

Introduction

In an earlier paper we reported on the promoting effect of a short duration moisture stress, induced by PEG given to pea stock plants at 38 W m^{-2} on the adventitious root formation on the cuttings, contrary to an inhibitory effect of the same treatment at 16 W m^{-2} (Rajagopal and Andersen 1979). It was suggested that the changes in the content of carbohydrates and endogenous level of ABA and ethylene might be involved in the differential rooting behaviour under the two irradiances with different degrees of stress. Exogenous application of ABA to the cuttings obtained from the stock plants grown at 16 W m^{-2} or 38 W m^{-2} resulted in a greater number of roots, especially in the cuttings from high irradiance (Rasmussen and Andersen 1979).

Moisture stress facilitates an increase in sugar content (see Hsiao 1973), accumulation of ABA (Wright and Hiron 1969) and high ethylene production (Aharoni 1978). Which of these factors is involved in the enhancement of adventitious root formation on the cuttings from the stressed plants grown at 38 W m^{-2} (Rajagopal and Andersen 1979)? This question was the main objective of the present investigation. We report here the changes in ψ_π, ABA level and ethylene evolution in pea plants stressed under two irradiances.

Abbreviations: ψ_π, leaf osmotic potential; ABA, abscisic acid; PEG, polyethyleneglycol; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.

Materials and methods

Growth of stock plants and stress treatments. Pea plants (*Pisum sativum* L. cv. Alaska) were grown under low or high (16 or 38 W m^{-2}) irradiances and on day 11 were subjected to different degrees of moisture stress for 2, 4 or 6 h simulated with PEG solutions of –3.0, –6.4 and –13.9 bar and prepared in the nutrient solution. The stress levels were designated as 'mild', 'moderate' and 'severe' respectively. Details of the method of growing stock plants and of stress treatments were described earlier (Rajagopal and Andersen 1980).

Determination of leaf osmotic potential, ψ_π. At the end of the 2, 4 or 6 h stress treatments applied to the stock plants on day 11, the ψ_π of the non-stressed and stressed plants were determined using a vapour pressure osmometer following the method described by Simmelsgaard (1976).

Extraction and purification of ABA. ABA was esti-

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mated in both the stressed and control plants at 2 or 6 h treatments. The leaves (10 g fresh weight) were weighed immediately and plunged into pre-cooled (–20°C) extraction solvent (methanol : chloroform : 2 *M* NH_4OH, 12:5:3: v/v/v). The extraction and purification procedures were the same as those described by Beardsell and Cohen (1975) and adapted by Bengtson *et al.* (1977). The recovery of *cis, trans*-ABA was estimated by adding known amounts (100–200 ng) of synthetic ABA (Fluka) to the unpartitioned extracts. Addition of ABA alone to the solvent mixture, without the plant extracts, served as the reference. The recovery was found to vary between 73 and 78%.

Methylation. The dried extracts were dissolved in 0.5 ml methanol and were methylated with freshly prepared diazomethane following the method of Powell (1964). After methylation the samples were dried and dissolved in a small volume (25 to 50 µl) of ethyl acetate and stored in the refrigerator.

Estimation of ABA with gas-liquid chromatography. Me-ABA was analysed quantitatively using a Hewlett Packard 7620A gas chromatograph with an electronic digital integrator. The flame ionization detector was employed. One µl of the sample was injected into a glass column 1.8 m × 2 mm i.d. packed with chromosorb G, 80–100 mesh QF, AW DMCS. The column temperature was 210°C, with injector and detector temperatures at 250°C and 200°C respectively. Nitrogen was used as the carrier gas at a flow rate of 25 ml min^{-1}. Hydrogen and air flow were 30 ml min^{-1} and 250 ml min^{-1} respectively. The sensitivity range was set at 10^3, with the recorder presentation on 10 mV. With this standard set up the retention time for Me-ABA was 9 to 10 min. Four injections were made for each sample.

Collection of ethylene from the intact plants. The evolution of ethylene from the non-stressed and stressed plants grown at 16 or 38 W m^{-2} was determined on day 11 at 2, 4 or 6 h immediately after the treatment. At the end of the treatment a batch of five plants was removed from the medium, nutrient solution or the PEG solutions, and the intact plants, with the roots wrapped in tissue paper, were placed upright in a 2-liter glass jar. The jar was then sealed with two layers of dental gum and tied properly to avoid any escape of ethylene gas. The jars were kept at the respective irradiances for 2 h.

Determination of ethylene production. At the end of the 2 h ethylene accumulation, the amount of ethylene was determined using a Hewlett Packard 7620A gas chromatograph fitted with a stainless steel column, 2 m × 2.4 mm i.d. and flame ionization detector. The column was packed with Poropak T, 80–100 mesh. Nitrogen was used as the carrier gas at a flow rate of 45 ml min^{-1}. The column temperature was set at 60°C and that of injector and detector at 90°C and 170°C respectively. The sensitivity range was 10^2, and the recorder presentation was on 0.1 mV. The retention time for ethylene was 85 to 90 s. Five ml of the gas sample was drawn from the jar and injected into the gas chromatograph with the aid of a

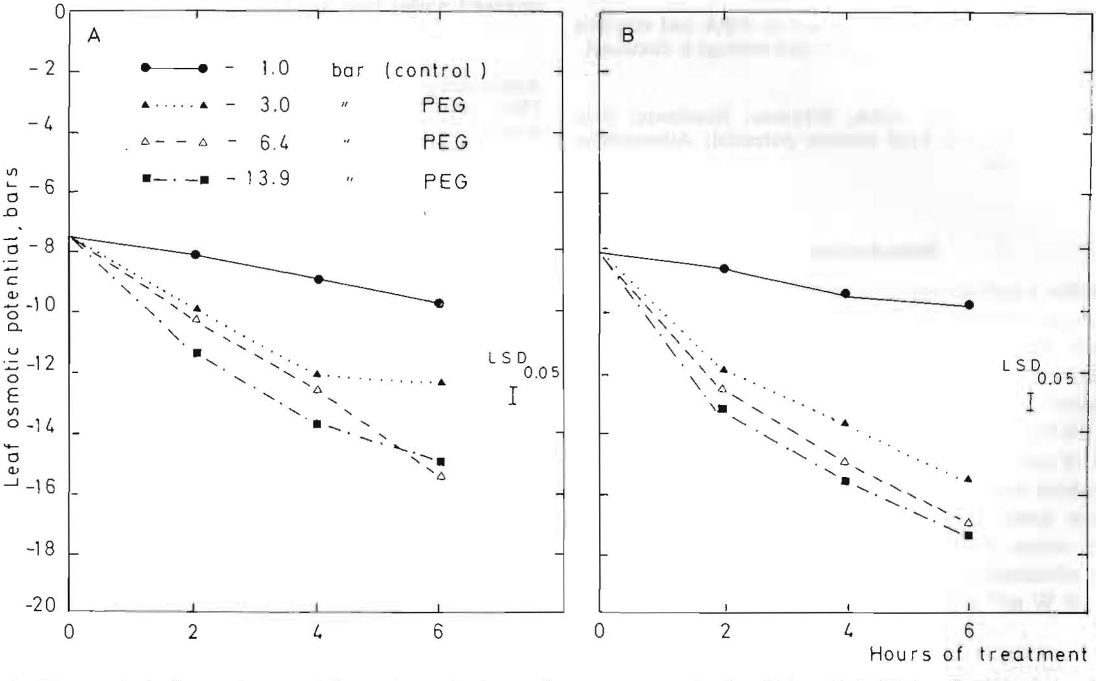

Figure. 1. *Changes in leaf osmotic potential, ψ_π, in stock plants of peas grown under low* (A) *or high* (B) *irradiance.* On day 11, they were stressed with different levels of PEG 6000 for 2, 4 or 6 h. Values are means of four replications.

Table 1. *Influence of moisture stress and irradiance on endogenous ABA content* (ng · plant^{-1}) *in pea leaves.* Stock plants were grown at 16 W m^{-2} or 38 W m^{-2} for 11 days. They were stressed with PEG 6000 of different osmotic potentials for 2 or 6 h. At the end of stress treatment, ABA was extracted from the leaves and estimated with GLC. See Materials and Methods.

Duration of stress,	Osmotic potentials of PEG (6000), bars							
	16 W m^{-2}				38 W m^{-2}			
h	−1.0 (control)	−3.0	−6.4	−13.9	−1.0 (control)	−3.0	−6.4	−13.9
2	290	619	591	325	178	1149	1108	380
6	182	369	201	149	248	435	453	197

semi-automatic injection pump. Four injections were made for each sample. Ethylene content was calculated from standard samples of pure ethylene (ALFAX, AB, Malmö, Sweden) which was diluted with air to appropriate concentrations.

Results

Leaf osmotic potential, ψ_π. The leaf osmotic potential of non-stressed plants showed little difference between low and high irradiances (Figure 1). The moisture stress imposed on the plants with different osmotic potentials of PEG resulted in lowering the ψ_π, the degree of reduction depending on the stress level, duration and irradiance level. For instance, in the 2 h stress under low irradiance the ψ_π was reduced from −8.1 bar in non-stressed plants to −9.9, −10.1 and −11.3 bar respectively with mild, moderate and severe stress. Under high irradiance the ψ_π declined from −8.4 bar in control plants to −11.9, −12.4 and −13.2 bar with the 2 h stress of three levels. Prolongation of stress led to a further fall in ψ_π under both levels of irradiance.

Endogenous ABA content. The ABA content in the non-stressed plants was slightly higher at 16 W m^{-2} than at 38 W m^{-2} after 2 h, but the reverse trend occurred at 6 h (Table 1). When the plants were subjected to different degrees of stress with PEG for 2 h under low irradiance ABA content was 619 ng, 591 ng and 325 ng plant^{-1} respectively, in mild, moderate and severe stress treatments, compared to only 290 ng plant^{-1} in non-stressed plants. Under high irradiance there was accumulation of ABA with the three levels of stress in the order of 1149 ng, 1108 ng and 380 ng plant^{-1} as against 178 ng plant^{-1} in the control. Prolongation of the stress for 6 h led to a marked decline in ABA content of all the stressed plants under both levels of irradiance; the content of ABA in the mildly- and moderately-stressed plants was still higher than in the non-stressed plants, whereas severely-stressed plants had less ABA than the control.

Ethylene production. There was little difference in the evolution of ethylene of non-stressed plants between low and high irradiance (4.2 and 4.6 nl plant^{-1} h^{-1}, Figure 2). However, under low irradiance the severe stress resulted in a significantly lower ethylene level (3.7 nl plant^{-1} h^{-1}) with little difference between 2 and 6 h, whereas with mild and moderate stress the ethylene level (4.4 and 4.3 nl plant^{-1} h^{-1}) was similar to or slightly higher than in the control plants. When the high irradiance-grown plants were stressed with −3.0, −6.4 and −13.9 bar PEG for 2 h, the production of ethylene was higher in the latter two treatments only (5.4 and 5.6 nl plant^{-1} h^{-1}), and with the 4 h treatment the effect still persisted (4.8 and 4.9 nl plant^{-1} h^{-1}); whereas by extending the stress for 6 h there was a significant fall in ethylene production with all the stress treatments, the decline being greater with the mild stress than the severe stress.

Discussion

Relationship between the leaf osmotic potential and ABA level. In general the moisture stress at 38 W m^{-2} facilitated greater accumulation of ABA than that at 16 W m^{-2}; the stress levels and duration showed variations in ABA level. For example, on imposing the stress for 2 h at 16 W m^{-1} there was a reduction in ψ_π of 1.8 to 2.0 bar from the control level (−8.1 bar) in the mildly- and moderately-stressed plants with an accumulation of ABA, 113% and 103% over the control. A further fall in ψ_π by 3.2 bar with severe stress reduced the ABA level (only 12% over the control) as compared to the other two stress treatments. Similarly, at 38 W m^{-2} the 2 h treatment of mild and moderate stresses enhanced the ABA level by 545% and 522% over non-stressed plants, with a corresponding fall in ψ_π by 3.4 and 3.9 bar (from the control level of −8.4 bar); a further decline in ψ_π by 4.7 bar with severe stress resulted in only 113% increase in ABA over control.

The threshold leaf water potentials for ABA accumulation are different for different species: −8 bar for maize and −8 to −10 bar for sorghum (Beardsell and Cohen 1975), −10 to −12 bar for *Ambrosia* (Zabadal 1974), −9.3 bar for excised wheat leaves (Wright 1977) and −7 to −9 bar in *Phaseolus vulgaris* (Walton *et al.* 1977). In the present investigation, the ABA content was low as long as

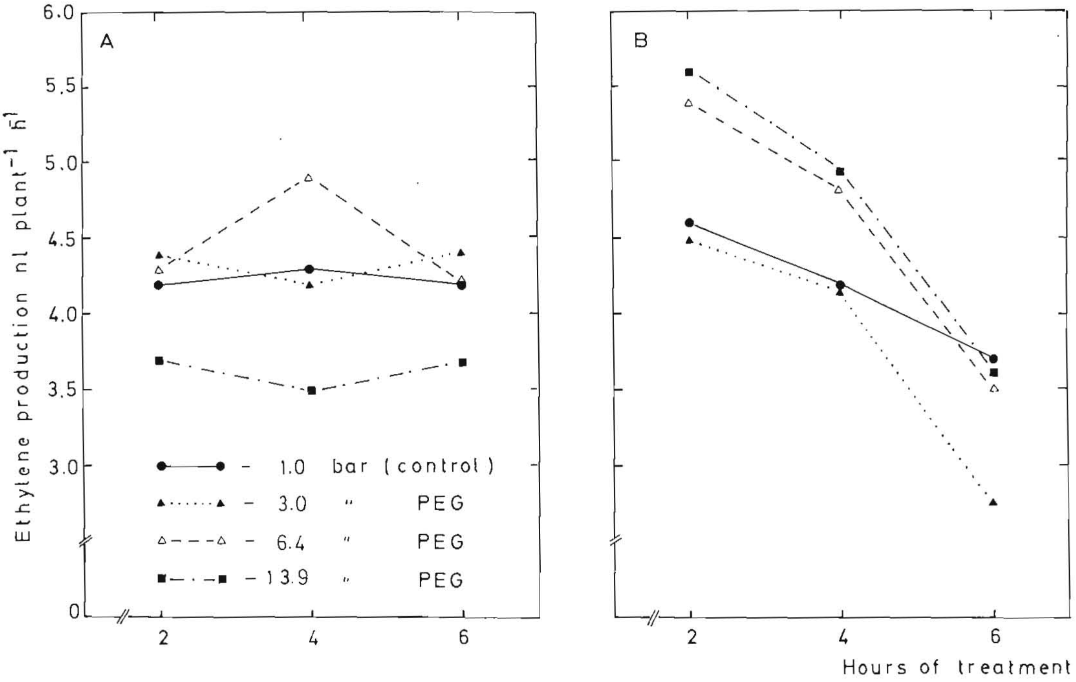

Figure 2. *Ethylene production by 'intact' pea plants grown under low* (A) *or high* (B) *irradiance and stressed with different levels of PEG 6000 for 2, 4 or 6 h.* Values are means of two determinations of five plants each.

the ψ_π was –8.1 to –8.4 bar (non-stressed), but as the potentials started declining to –9.9 to –10.1 bar (at 16 W m^{-2}) or –11.9 to –12.4 bar (at 38 W m^{-2}) the ABA level increased by 103 to 113% and 522 to 545% over control. Thus, a threshold leaf osmotic potential around –9 to –12 bar, depending on the irradiance level, occurred in peas. This sudden spurt in ABA level at ψ_π lower than –8.5 bar indicates that the ABA-accumulating system was "switched-on", as suggested by Zabadal (1974), who observed the same trend in the critical range of –10 to –12 bar in *Ambrosia*. However, he found a continuous increase in ABA level with the decreasing water potential range of –12 to –24 bar, whereas in peas there was a marked reduction in ABA content with lowering ψ_π as indicated by the severely-stressed plants under both levels of irradiance. Wright (1977) also found a threshold potential of –9.3 bar in excised wheat leaves. However, his experiments indicated a gradual increase in ABA levels with decreasing leaf water potential, unlike the sudden "switch-on" mechanism found by Zabadal (1974) which was also indicated in our experiments. An 'abrupt' increase in ABA content with the fall in ψ_π was also reported for sorghum and maize (Beardsell and Cohen 1975). Walton *et al.* (1977) also observed a 'sharp' increase in ABA level over a relatively narrow leaf water potential range in *Phaseolus vulgaris*. The decrease in ABA content with the ψ_π lower than –14 to –15 bar was indicated by the prolonged stress (6 h) of all the levels under both levels of irradiance. Thus, there seems to be a threshold osmotic potential not only for the accumulation of ABA, but also for its decline as observed within the experimental limits of the present study.

A 2 h treatment of –4 bar PEG 6000 applied to the 11-day-old pea plants resulted in a tenfold increase in ABA in both roots and leaves (Walton *et al.* 1976). In the present study also in the 2 h treatment of –3.0 and –6.4 bar PEG there was a tenfold rise in ABA level at 38 W m^{-2}, but only a threefold rise at 16 W m^{-2}. Thus, the irradiance level influenced the rate of accumulation of ABA for the same duration of similar treatment. In wilted pea shoots Simpson and Saunders (1972) observed a fourfold increase in ABA in dark-grown plants compared to a ninefold increase in light-grown ones.

Furthermore, variations in ABA level are reported not only for different plant species but also for the same plant, depending on the nature, degree and duration of stress treatments as well as the plant age. For instance, Dörffling *et al.* (1974) reported a twentyfold increase in ABA in 4-week-old pea shoots wilted for one week. When the excised pea epicotyls were wilted for 8 h, there was a sevenfold rise in ABA level (Simpson and Saunders 1972). ABA content increased ten times in both roots and leaves of peas when stressed for 2 h with –4 bar PEG (Walton *et al.* 1976). Our results with peas also show variations in ABA content between the treatments and irradiances.

Relationship between the leaf osmotic potential and ethylene production. Though the ψ_π was lowered from –8.1 bar in the non-stressed plants to –11.3 bar in the severely-stressed plants during the 2 h treatment at 16 W m^{-2}, there was no appreciable change in the ethylene production. On the other hand, the 2 h stress treatment at 38 W m^{-2} resulted in greater reduction in ψ_π from –8.4 bar in control plants to –13.2 bar with increasing degree of stress. This corresponded to higher ethylene production, especially with moderate and severe stress (17.5 and 23.5%), than in the case of the non-stressed plants. Prolonging the stress to 6 h led to further reduction in ψ_π under both levels of irradiance, but ethylene production declined markedly only under high irradiance.

In excised wheat leaves a leaf water potential of –12 bar was found to be optimal for ethylene biosynthesis by Wright (1977). He found an increasing production of ethylene with an increasing degree of stress and duration until 11 h, after which the production levelled off. When the water potential of the medium was reduced from 0 to –6 bar the ethylene level increased in the leaves of *Vicia faba* (Hall *et al.* 1977). In cotton petioles ethylene production started after the potential reached –20 bar (McMichael *et al.* 1972). In the present study, ethylene production increased significantly only in plants grown at 38 W m^{-2} and stressed for short duration (2 h) with a reduction of –11.9 to –13.2 bar in ψ_π. Prolonged stress at 38 W m^{-2} and all the stress periods at 16 W m^{-2} failed to enhance ethylene evolution. This indicates that the level of irradiance influences ethylene production. For the same degree and duration of stress, the plant response in terms of ethylene production was different under two irradiances: An exposure of low irradiance-grown plants to severe stress for 2 h reduced the ψ_π to –11.3 bar and decreased ethylene production by 11.5% of non-stressed plants, while the similar treatment for the same duration under high irradiance resulted in a further reduction of ψ_π to –13.2 bar, but increased the production of ethylene by 23.4% over the control. The fall in ethylene production observed in some of the treatments with prolonged stress is in accordance with the findings of El-Beltagy and Hall (1974), who, in the leaves of *Vicia faba* under water stress, also found a peak in ethylene production during 24 h followed by a decline for another 2 days.

Relationship between ABA and ethylene levels. A significant accumulation of ABA in the stressed plants occurred only at 38 W m^{-2} with the 2 h treatment, when ethylene production also registered a marked increase compared to non-stressed plants, whereas at 16 W m^{-2} ABA alone increased. An increase in the level of both ABA and ethylene under drought or water-logged conditions was reported in *Vicia faba* (Hall *et al.* 1977). Which of the two accumulate first in response to the short duration stress at 38 W m^{-2}? With decreasing ψ_π from –8.4 bar (non-stressed plants) to –11.9 bar (mild stress) ABA accumulated fivefold, but ethylene did not increase, while a further reduction just by 0.5 bar, *i.e.* to –12.4 bar (moderate stress) triggered the ethylene production, with ABA maintaining almost the same high level. However, when the ψ_π fell by another 0.8 bar, *i.e.* to –13.2 bar (severe stress) ethylene production increased further, but a marked decline in ABA level occurred. Thus ABA responded first to the decreasing ψ_π, while ethylene production followed only with a further decline in ψ_π. In excised wheat leaves the rate of increase in ABA level preceded that of the ethylene production (Wright 1977). The investigation of Wright (1977) and of Aharoni (1978) disproved the assumption that the stress-induced ethylene evolution stimulated the accumulation of ABA. Mayak and Halevy (1972) found a rise in ethylene evolution followed by an increase in the level of ABA in rose petals. A close examination of the changes in ψ_π, ABA and ethylene with the 2 h stress at 16 and 38 W m^{-2} indicates that there was a difference of –3 to –4 bar in ψ_π between the accumulation of ABA and ethylene production, the latter following the former, which is in agreement with the observations of Wright (1977) and Aharoni (1978).

Relationship between the levels of ABA, ethylene production and adventitious root formation. Rajagopal and Andersen (1979) reported a significant increase in the formation of adventitious roots on cuttings obtained from stock plants grown and stressed for 2 h at 38 W m^{-2}. Both ABA and ethylene were also higher in the 2 h stressed plants with decreasing ψ_π at 38 W m^{-2} than in the non-stressed ones (Figures 1 and 2; Table 1). Thus it appears that ABA and ethylene were involved in enhancing the rooting of cuttings, though it is difficult to say which was more effective. Nevertheless, a critical comparison of the situation obtained with the 2 h treatments at 16 W m^{-2} reveals that ABA, but not ethylene, increased under the influence of stress and that the rooting was less than in the non-stressed ones. Either the low content of ABA or the lack of ethylene production, or both, explains the poor rooting in this case. Also, the low levels of both ABA and ethylene with prolonged stress, 6 h, under both levels of irradiance might be the reason for the poor adventitious root formation at 6 h stress.

Both the promoting effect, and the lack of effect or the inhibiting effect on adventitious root formation were attributed to ethylene in different plant species depending on the nature of material used: intact or excised, leafy or non-leafy cuttings, etiolated or non-etiolated segments (see Andersen 1975). Furthermore, Andersen (1975) emphasized the importance of the medium for dissolving ethephon, as well as the influence on the adventitious root formation of the concentration and irradiance level under which the stock plants were grown. Using the mixtures of IAA, IBA and ethephon, which have differential effects on ethylene production and promotion of rooting, on etiolated mung bean hypocotyl Batten and Mullins (1978) concluded that ethylene is not directly involved in the adventitious root formation. In a previous study of the pea cutting system (Andersen 1975), exogenous ethylene was found to have a differential effect on root formation depending on time of application, concentration and the irradiance applied to stock plants. Rooting was promoted by application 2 or 3 days after cutting under high irradiance. Reduction of root formation was observed with pre-cutting application, high concentration and low irradiance applied to stock plants. Since the ethylene production in the cuttings was not followed in the present investigation, it is not possible completely to rule out the participation of ethylene in the observed results (Rajagopal and Andersen 1980), but it appears unlikely that the observed small changes in ethylene production due to the imposed stress should have any implication for the rooting of these cuttings.

The role of ABA on the rooting of cuttings has been reported for many species (Chin *et al.* 1969, Rajagopal *et al.* 1971). Rasmussen and Andersen (1979) found a promoting effect of ABA (10^{-6} to $10^{-3}M$) on the rooting of pea cuttings, the effect being more pronounced at 38 W m^{-2} (111% over control) than at 16 W m^{-2} (64% over control). In the present study, ABA content in the plants stressed for 2 h with –3.0 and –6.4 bar PEG at 38 W m^{-2} was two- to threefold higher than under the similar treatment for the same duration at 16 W m^{-2}; adventitious root formation was enhanced only in the former case (Rajagopal and Andersen 1979).

It is thus evident that the stress-induced and light-mediated increase in the adventitious root formation during the 2 h stress at 38 W m^{-2} (Rajagopal and Andersen 1979) is due to an enhanced level of ABA which might have caused metabolic changes in the cuttings during the root initiation and development stages.

The results reported in this series of papers have established the influence of irradiance and the stress levels as well as the duration of stress treatment applied to stock plants on the formation of adventitious roots of pea cuttings. The stimulating effect of ABA, both as exogenous treatment and enhanced endogenous level as a result of stress treatments, on the rooting of cuttings depended on the level of irradiance under which the stock plants were grown. From these experiments we could once again observe the significance of stock plant growth conditions for studies on root regeneration.

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