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Effect of *Eucalyptus* Growth Regulators on the Water Loss from Plant Leaves

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ABSTRACT

Three closely related growth regulators (G) that are present in some myrtaceous plants were examined for possible anti-transpirant effects. The bioassay material involved cuttings of mung bean and *Eucalyptus rupicola*. Stomatal resistance was determined by a diffusion porometer. Water loss was equated with water uptake by the cutting over a 24-hour period.

In both bioassays, G reduced water loss. The reduced water loss was associated with stomatal closure. This anti-transpirant effect of G was five to ten times less than that of abscisic acid. The stomatal resistance to the diffusion of water vapor from mung bean leaves increased within 1 hour of application of G. Marked stomatal closure occurred after 6 hours when 5 to 7 micrograms of G had accumulated in the leaves.

These results and earlier evidence, suggest that G growth regulators are involved in the water economy of *Eucalyptus* and perhaps other related genera.

Three closely related growth regulators designated as G substances or G[1], occur in leaves of *Eucalyptus grandis* and some other myrtaceous plants (3). The structure (1, 9, 11) of G_1, G_2, and G_3 is given in Figure 1. They occur in about equal concentrations that are especially high (7,500 μg/g) in adult leaves of *E. grandis*. Their chemical synthesis has been achieved (2).

The three forms of G are equally active in various bioassays: cress seed germination (11) is inhibited at high concentrations; coleoptile growth (3) and rooting of mung bean cuttings (3) are inhibited at high concentrations but promoted at low concentrations. Water uptake by cuttings was often observed to be less in the presence of G than in control plants in the mung bean-rooting bioassay. This observation agrees with earlier indications that high endogenous levels of G are associated with the reduced wilting of adult *E. grandis* leaves when exposed to water stress (10). The present paper examines the effect of G on the water uptake and stomatal opening in mung bean and in a selected *Eucalyptus* species, *E. rupicola*.

MATERIALS AND METHODS

Seeds of mung bean (*Vigna radiata* [L] Wilczek, previously *Phaseolus aureus* Roxb. cv. Berken) were purchased locally. These were soaked for 3 h in a weak fungicidal solution (0.1% Captan) at about 25 C. Sowing depth was 2 cm in pots of Perlite. The pots were maintained at 25 C for 10 days. Germination and stem elongation occurred during the first 5 days when exposure to dim light (0.1 w m^{-2}), except during short periods for watering and for one application of Hoagland solution at day 3, produced etiolated plants between 10 and 15 cm in height. At day 5, the plants were exposed to either 12 h photoperiod (17.5 w m^{-2}) in a growth cabinet or natural daylength in a shaded glasshouse.

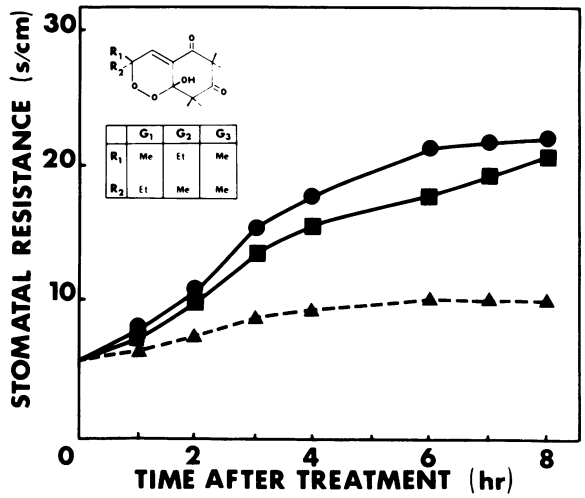

FIG. 1. The stomatal resistance to diffusion of water vapor (s/cm) in mung bean cuttings cultured in water (▲); 5×10^{-4} M G (●); and 1×10^{-4} M G (■). Seedling cuttings prepared as described under "Materials and Methods," were allowed to adjust to the cabinet conditions for 24 h before being transferred to test solutions. The experiment involved 90 cuttings; 30 each in the water control; 5×10^{-4} M G; and 1×10^{-4} M G. Five cuttings from each test solution were used for recording stomatal resistance at 1, 2, 3, 4, 6, and 8 h. Cuttings were discarded after use. A new group of cuttings was used for each time interval. Insert shows the structure of the three known forms, G_1, G_2, and G_3. (1)

Ten-day-old seedlings having fully expanded primary leaves were selected for uniformity in height (≃15 cm) and leaf size. The hypocotyls were excised 5 cm below the cotyledons. The excision was made under water to prevent entry of air. The seedling cuttings were cultured in narrow mouth glass vials (4 cm long, 0.8 cm diameter) that were completely filled with 3 ml of test solution at the beginning of the experiment. The cuttings were kept in growth cabinets at 25 C and 12 h fluorescent light (17.5 w m^{-2}) with a gentle air movement.

The treatments involved a range of concentrations of G in water from 5×10^{-4} M to 5×10^{-6} M. Two concentrations of ABA (1×10^{-4} M and 1×10^{-5} M) were included for comparison. The volume of liquid taken up by the cutting was replenished by water, not solution. This procedure was based on the rapid initial uptake of G by the cutting (*cf.* Fig. 2).

It was found desirable to protect the cuttings with a transparent plastic cover to reduce wilting during the first 24-h period. The high humidity under the cover reduced solution uptake to <0.3 ml/cutting. At the end of this adjustment period, the plastic cover was removed and water was added to bring the upper meniscus to

[1] Abbreviation: G: growth regulators.

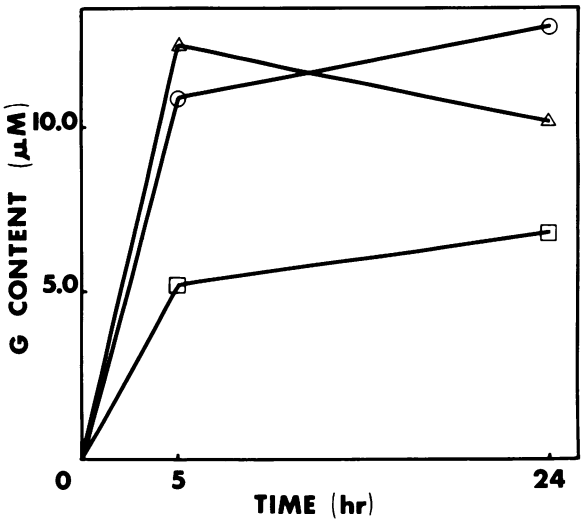

FIG. 2. The content (μg) of G in leaves (□); hypocotyls (△); and epicotyls (○) of mung bean seedling cuttings at 5 and 24 h after treatment in 5×10^{-4} M G. Each point represents the average of three replicates with 20 seedlings each.

the top edge of the vial.

During the 5 days following the 1-day adjustment period, the uptake of solution was recorded every 24 h by refilling the glass vials using a graduated hypodermic syringe. As the fresh weight of cuttings was not markedly affected by treatment and the surface evaporation in such narrow mouth vials was uniform (< 0.05 ml/vial), the solution uptake from the vials was regarded as the water loss from the leaves. The results presented in Table I are the mean of five replicates and the experiment has been repeated several times with similar results.

Cuttings of *E. rupicola* Johnson and Blaxell, were collected from the adult trees growing in the Botany Nursery, Australian National University, Canberra. The adult leaves of this species contain the low amounts of G that are commonly found in *Eucalyptus* other than in adult *E. grandis* (3). This species of *Eucalyptus* was particularly suitable for the bioassay since the narrow leaves do not wilt readily and they are about the size of primary leaves of mung bean seedlings. Twelve-cm-long cuttings with three pairs of apical leaves similar in shape and size were used in place of mung bean cuttings.

The stomatal resistance to the diffusion of water vapor from the lower side of mung bean leaves was recorded using a ventilated diffusion porometer (13), calibrated as described by Kanemasa *et al.* (5).

The sample of G used throughout the experiments was of a high purity as confirmed earlier (3). The procedure for isolation of G involved methanol extraction of adult leaves of *E. grandis*, partitioning with cyclohexane and chromatographic purification using a cellulose column with ethylene glycol-water (4:1) as the stationary phase and cyclohexane as the mobile phase.

Isolation and quantitative assay of G in mung bean experiments followed established procedure (3). This involved methanol extraction, partitioning with chloroform and purification on three successive silica gel plates with different solvents. Those compounds that cochromatographed with standard G were identified by NMR and assayed by absorption at 240 nm.

RESULTS

Concentrations of G between 5×10^{-4} M and 5×10^{-6} M reduced the water loss from mung bean cuttings (Table I). The magnitude of the response depended on concentration with maximum reduction of water loss occurring at 5×10^{-4} M G. The reduced water loss persisted for at least 5 days although initial concentrations of G above 1×10^{-5} M maintained a significant reduction in water loss until after the 5th day. Between days 1 and 2, 1×10^{-4} M ABA reduced water loss to 34% of control compared with 43% for 5×10^{-4} M G (Table I). On subsequent days, differences between these two treatments became less obvious with the respective reductions to 43 and 46% at days 4 and 5 involving nonsignificant ($P > 0.02$) differences between the means. At days 4 and 5, 1×10^{-4} M G reduced water loss to 73% of control compared with the 43% for ABA. The effectiveness of G as an anti-transpirant in the mung bean bioassay was thus 5 to 10 times less than that for ABA.

As shown in Table II, the uptake of G increased the stomatal resistance to the diffusion of water vapor. High concentrations of G (5×10^{-4} M and 1×10^{-4} M) were comparable to ABA (1×10^{-4} M) in causing marked stomatal closure. The partial closure at 1×10^{-5} M ABA was approximately equivalent to 5×10^{-5} M G. Lower concentrations of G affected the stomatal resistance with the increase associated with 5×10^{-6} M G being maintained for

Table I. *Mean Water Loss per Cutting (ml ± SE) from Leafy Cuttings over a 24-hour Period*

Successive 24-h periods between days 1-2, 2-3, 3-4, and 4-5 after an initial 24-h adjustment period. Figures in brackets indicate water loss as a percentage of the appropriate control. Mung bean cuttings were similar to those in Figure 1. *E. rupicola* cuttings were from adult shoots

Molar Concentration	No. of Days			
	1-2	2-3	3-4	4-5
Mung bean				
Water control	1.48 ± 0.17 (100)	1.04 ± 0.05 (100)	0.97 ± 0.05 (100)	0.97 ± 0.05 (100)
Mung bean				
G, 5×10^{-4}	0.64 ± 0.05 (43)	0.52 ± 0.01 (50)	0.51 ± 0.02 (53)	0.45 ± 0.03 (46)
1×10^{-4}	0.89 ± 0.03 (60)	0.74 ± 0.06 (71)	0.73 ± 0.04 (75)	0.71 ± 0.04 (73)
5×10^{-5}	0.95 ± 0.13 (64)	0.78 ± 0.04 (75)	0.80 ± 0.05 (83)	0.77 ± 0.05 (79)
1×10^{-5}	1.22 ± 0.15 (82)	0.90 ± 0.03 (87)	0.93 ± 0.04 (96)	0.86 ± 0.05 (89)
5×10^{-6}	1.29 ± 0.07 (87)	0.95 ± 0.03 (91)	0.96 ± 0.03 (99)	0.95 ± 0.03 (98)
Mung bean				
ABA, 1×10^{-4}	0.50 ± 0.02 (34)	0.46 ± 0.02 (44)	0.44 ± 0.03 (45)	0.42 ± 0.01 (43)
1×10^{-5}	0.77 ± 0.07 (52)	0.73 ± 0.04 (70)	0.65 ± 0.02 (67)	0.61 ± 0.02 (63)
E. rupicola				
Water control	0.99 ± 0.03 (100)	1.05 ± 0.05 (100)		
E. rupicola				
G, 5×10^{-4}	0.81 ± 0.02 (82)	0.84 ± 0.04 (85)		

Table II. *Stomatal Resistance in Mung Bean Seedlings as Affected by Varying Concentrations of G and ABA at 1 and 3 Days*

Molar Concentration	Day	
	1	3
	s/cm ± SE	
Water control	12.2 ± 1.0	10.3 ± 0.4
G, 5×10^{-4}	[a]	[a]
1×10^{-4}	[a]	[a]
5×10^{-5}	22.7 ± 3.6	21.2 ± 1.7
1×10^{-5}	17.4 ± 3.3	16.7 ± 3.0
5×10^{-6}	16.8 ± 1.4	13.2 ± 0.3
ABA, 1×10^{-4}	[a]	[a]
1×10^{-5}	23.8 ± 1.3	18.5 ± 1.9

[a] Stomatal resistance high enough to indicate complete stomatal closure.

up to 4 days. The increased stomatal resistance can be detected 1 h after the cuttings were immersed in G solution (Fig. 1). After 5 h, the stomatal resistance of cuttings in 5×10^{-4} M G solution increased to twice that of the control. At this concentration, the amount of G that had accumulated in the two primary leaves after 5 h was 5.3 μg (Fig. 2). With marked stomatal closure at 24 h (*cf.* Table II), the amount of G in these leaves was 6.9 μg. Both hypocotyl and epicotyl tissue had higher G content than the leaf tissue after 5 and 24 h (Fig. 2).

A solution of G at 5×10^{-4} M decreased the water loss from *E. rupicola* cuttings to about 20% of control (Table I). The amount lost from each cutting over a period of 24 h was of the same order as that lost by mung bean cuttings over the same period but the effect of G on percentage reduction in water loss appears to be less for *Eucalyptus* cuttings than for mung bean cuttings.

DISCUSSION

The increased stomatal resistance of leaves of mung bean cuttings in G solution explains their reduced water loss. Only small amounts of G appear necessary to decrease stomatal aperture. The response at 5×10^{-6} M is especially interesting as it indicates that the effective concentration of G is markedly lower than that for chemicals such as acetyl salicylic acid, which does not affect water loss at concentrations below 1×10^{-3} M (6).

Although increased stomatal resistance can be detected 1 h after the cuttings are immersed in G solution, marked stomatal closure occurs after 6 h when 5.0–7.0 μg G has accumulated in mung bean leaves. This amount of G in leaf cells thus appears to be the minimum to effect marked stomatal closure.

The time course for accumulation of effective amounts of G in cuttings appears to be one factor which results in maximum water loss during the first 24 h. This interpretation does not apply in control plants. Thus some further factor is presumably involved in the general decrease in water loss over the first 2–3 days. Stomatal resistance also decreases over this period. Such an inverse physiological relationship probably arises because readings of stomatal resistance were taken over a short interval at midmorning whereas those for water loss refer to a 24-h period. Further information is needed to help explain these interesting relationships.

Some small trials indicate that spray applications of G also increase stomatal resistance and thereby decrease water loss. Presumably, spray applications of G and uptake of G by the transpiration stream both allow accumulation of comparable amounts of G in leaf tissue. While the mechanism involved in stomatal closure by G is not known, some preliminary results indicate that G affects rubidium (^{86}Rb) uptake by *Avena* and red beet tissue (Dhawan, unpublished data). This result implies that G may have an effect on membrane transport, an effect claimed for ABA (4, 8, 12).

Although the anti-transpirant activity of G in mung bean and in *E. rupicola* implies that high concentrations of G in adult leaves of *E. grandis* may have a similar role, an experimental approach along these lines has encountered several difficulties. One of these was that exogenous G is probably ineffective with the high endogenous G content of adult *E. grandis* leaves (3). Conversely, juvenile leaves containing small amounts of G (3) may metabolize exogenously applied G.

A further problem is the absence of transport of G between adult and juvenile tissue in this species (10). Practical problems included a high degree of natural variability in water uptake by cuttings of *E. grandis* and the tendency for the large leaves to wilt during the anti-transpirant bioassay. Some of these points can be further examined when radioactive synthetic G is available.

Despite these difficulties, earlier work (10) has shown that low temperature induced wilting in *E. grandis* was less for adult leaves (high G) than for juvenile leaves (low G) and also that spray applications of G were effective in preventing wilting of the juvenile leaves but less so than ABA. The present experiments confirm this anti-transpirant effect of G in mung bean leaves. That G reduces water loss also in *E. rupicola*, a myrtaceous plant having low levels of endogenous G, is further evidence that G may be involved in the water economy of *Eucalyptus*. Anti-transpirant activity is thus confirmed as one of the possible roles envisaged for G in *Eucalyptus* and other Myrtaceae (3, 7).

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