Plant Physiol. (1979) 63, 1044–1048
0032-0889/79/63/1044/05/$00.50/0

Abnormal Stomatal Behavior and Hormonal Imbalance in *flacca*, a Wilty Mutant of Tomato

V. EFFECT OF ABSCISIC ACID ON INDOLEACETIC ACID METABOLISM AND ETHYLENE EVOLUTION

Received for publication July 6, 1978 and in revised form January 12, 1979

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ABSTRACT

The wilty tomato mutant *flacca*, the normal cultivar *Lycopersicon esculentum* Mill. Rheinlands Ruhm, and abscisic acid-induced phenotypic revertants were compared with respect to ethylene evolution, activity of tryptophan aminotransferase, and [1-^{14}C]indoleacetic acid decarboxylation.

The level of ethylene evolution was higher in *flacca* plants than in the normal cultivar. Ethylene evolution was reduced to the wild type level in abscisic acid-induced phenotypic revertants and to a lesser extent in mutant plants grown under humid conditions. Leaf epinasty, which characterized *flacca* plants in the present experiments, did not appear in absciscic acid-treated mutant plants, but did appear under high humidity. Tryptophan aminotransferase activity, similar to ethylene evolution, was higher in *flacca* plants and was reduced to the normal level by abscisic acid treatment. Indoleacetic acid decarboxylation was similar in mutant and normal plants, but was increased by abscisic acid treatment. The relationships among ethylene, auxin, and the morphological symptoms which characterize the mutant are discussed.

The modification of hormone balance in the wilty tomato mutant *flc*[1] is apparently responsible for the abnormalities of its stomatal behavior and morphology. This modification includes a reduced ABA level and an increase in the concentration of auxin-like substances and kinetin-like activity (13, 16). The reduced ABA level was suggested as the first of these three hormonal changes (7, 17) and seems to be the primary metabolic defect of this mutation.

Following ABA treatment, the mutant completely reverts phenotypically to the normal type with regard to stomatal behavior (7), transpiration rate (7), root resistance to water flow (14), kinetin-like activity (16), as well as peroxidase (15) and RNase (9) activities. Similarly, the mutant morphological symptoms characteristic of excess of auxin activity (7), *e.g.* leaf epinasty, swelling of the upper stem, rooting along the stem, and pronounced apical dominance (12, 13), do not appear in ABA-treated *flc* plants.

Abeles (1) has reviewed several studies which indicate that auxin effects on epinasty, root initiation, stem swelling, and growth inhibition are mediated by ethylene production in many plant species. Auxin induces ethylene production, and ethylene in turn induces the morphological symptoms. Ethylene production is also known to be induced by water stress (3, 4, 8, 22).

In view of the knowledge reviewed above, we attempted to clarify in this work the following questions. (*a*) Does ABA affect auxin level through inhibition of IAA synthesis and/or enhancement of its breakdown? For this purpose, normal, mutant and ABA-treated mutant plants were compared with respect to the activity of Trp-aminotransferase, which is the first enzyme in the prevalent IAA biosynthetic pathway (10, 19, 21), and [1-^{14}C]IAA decarboxylation, which is the first step in IAA breakdown. (*b*) Are the mutant morphological symptoms, characteristic of auxin excess, mediated by ethylene? Ethylene evolution was, therefore, compared in normal, mutant, and ABA-treated mutant plants, as it was assumed that if ethylene is a mediator hormone in auxin action, its production should be higher in the mutant than in the normal genotype. Accordingly, ethylene production was expected to decrease in ABA-treated mutant plants, in which the symptoms mentioned above do not appear. (*c*) Does ABA affect ethylene production through its effect on water balance? This aspect was studied by comparing ethylene evolution in normal and mutant plants grown under different humidities. Under normal greenhouse humidity (60–70%), mutant and normal plants differ in both ABA and water content (9, 13, 17). Under high humidity (90–100%), these plants differ in ABA level only, as the water balance in the mutant plants is improved without a concomitant increase of ABA level (9).

MATERIALS AND METHODS

The mutation *flc* was induced by x-ray irradiation of seeds of the normal tomato *Lycopersicon esculentum* Mill. cv. Rheinlands Ruhm (11), which was used as the control genotype in the present work. Plants were grown in the greenhouse in aerated, 0.5 Hoagland solution (6).

ABA Treatment. Normal and mutant plants were sprayed daily, from the two-leaf stage, with a solution of 10 μg/ml ABA solution containing 0.01% (v/v) Tween 80. Normal and mutant plants treated with ABA were designated RR_{ABA} and flc_{ABA}, respectively. Control plants were similarly sprayed with distilled H_2O containing 0.01% (v/v) Tween 80.

High Humidity. While most of the plants were grown under usual greenhouse conditions with temperature of 20 to 30 C and 60 to 70% RH (designated hereafter as "normal" humidity), one

[1] Abbreviations: DMAC: dimethylaminocinnamaldehyde; *flc*: *flacca*; IAAld: indoleacetaldehyde; IPyA: indolepyruvic acid; KG: α-ketoglutaric acid; RR: *Lycopersicon esculentum* cv. Rheinlands Ruhm; Trp: tryptophan.

group of plants was grown from the three-leaf stage in a polyethylene chamber under 90 to 100% RH (designated as "high" humidity). The RH was determined by hygrometer. Plants bearing 13 to 15 leaves and one to two influorescence primordia (designated "mature plants") were used in all of the experiments. For ethylene evolution determinations, plants bearing five to seven leaves (designated "young plants") were also used.

Trp-Aminotransferase Activity. The upper third of the plant was used for enzyme preparation. The activity was assayed by determination of Glu and IPyA produced by transamination between Trp and KG. The method described by Wightman and Cohen (20) for the extraction and purification of the enzyme and for determination of Glu was adapted with some modifications. The reaction system consisted of 1 volume of 0.03 M L-Trp, 0.02 M KG, 0.1 mM pyridoxal phosphate, and 1 volume of enzyme preparation in 0.1 M phosphate buffer (pH 8.5). Boiled enzyme and reaction system without KG were used as blanks in a few experiments.

The reaction system was incubated at 35 C for 2 h. One-ml aliquots, taken at 0 to 2 h, were added to 3 ml of acidified absolute ethanol. The amount of Glu was determined after chromatographic separation of 200-μl aliquots of the ethanolic solution on Whatman No. 1 chromatography paper with the solvent system 1-butanol-pyridine-water (1:1:1, v/v). The dried paper was dipped in ninhydrin-cadmium acetate reagent (2), and the color was developed over ethanol fumes for 30 min at 60 C. The Glu band was eluted with 5 ml of methanol for 2 h, and absorption of the eluate was determined by a Bausch & Lomb Spectronic-20 at 500 nm. Standard solutions of 1 to 10 μg Glu were run with each sample.

For IPyA determination, aliquots of the acidified ethanolic solution were spun down, and the supernatant was read by the Unicam SP-700A, a double beam spectrophotometer, in the range of 305 to 370 nm. IPyA concentration was determined from the differential readings at 340 and 360 nm. Actual concentrations were calculated, using a standard curve prepared from synthetic IPyA (Aldrich Chemical Co.), which exhibited linearity in the range of 1 to 20 μg/ml. No other component in the reaction system except IPyA showed any noticeable absorption changes within this range.

Neutral and acidic ether fractions of the reaction system were extracted, and the indole compounds were analyzed essentially according to Wightman and Cohen (20). The extraction pH was set at 3.0, and paper chromatography was performed on Whatman No. 1, using the organic phase of the solvent system benzene-glacial acetic acid-water (2:2:1, v/v). The paper was developed with 0.1% DMAC in acetone-HCl (19), heated for 2 min at 60 C, and the colored spots were measured with a Photovolt densitometer with a 570 nm filter. The area on the recorder paper chart corresponding to the colored spots was determined by weighing the cut area by a Sartorius analytical balance with 0.01-mg accuracy and converting to area units from a standard curve.

Protein content of the enzymic preparation was determined by estimation of nitrogen content with a Technicon autoanalyzer after Kjeldhal digestion and multiplying the N values of 6.25.

Decarboxylation of [1-^{14}C]IAA. Discs, 9 mm in diameter, were cut from the third leaf from the top of the plant. Groups of two discs each were transferred to sterilized scintillation vials containing 1 ml H_2O. Five μl (about 20,000 cpm) [1-^{14}C]IAA (The Radiochemical Centre, Amersham, England, 52 mCi/mmol) were added to each vial. A smaller vial containing 0.3 ml 0.1 N NaOH was placed in each scintillation vial as a CO_2 trap. The bigger vials were tightly sealed with stoppers and kept for 24 h under the same conditions as described by Epstein and Lavee (5). At the end of the experiment, the stoppers were removed, the NaOH from the small vials was transferred into scintillation vials, and 10 ml scintillation fluid (4 g PPO and 0.2 g POPOP in 1 liter toluene-Triton-100, 2:1, v/v) were added to each vial. Radioactivity was determined in a Packard liquid scintillation spectrometer. The control vial did not contain leaf tissue. No measures were taken in the present experiments to sterilize the leaf discs, because *flc* leaves are highly sensitive to detergents and because plate counts made of leaf discs showed no difference in bacterial contamination between *flc* and RR.

Ethylene Evolution. The upper part (bearing four to five leaves) of the plant was placed in a 500-ml tightly sealed glass jar containing 5 ml water. After 24 h, 1 ml of air was drawn from the jar with a syringe, and ethylene concentration was determined by a Packard 7400 gas chromatograph equipped with a hydrogen flame ionization detector and an activated alumina column. No ethylene was detected in air samples from the jars at zero time and from a jar which did not contain plant material and kept for 24 h under the experimental conditions.

RESULTS

Trp-Aminotransferase Activity. The amount of Glu produced by transamination of Trp was greater in *flc* than in RR plants (Table I). ABA-treated *flc* plants showed lower Trp-aminotransferase activity than *flc* plants. The same tendency was shown when IPyA, formed in the reaction, was examined.

Analysis of the acidic and neutral indoles formed in the reaction revealed five DMAC-positive bands on chromatograms developed in benzene-glacial acetic acid-water (2:2:1, v/v) (Table II). Three of these bands, which corresponded on an R_F basis to IPyA, IAA, and IAAld, showed a similar trend in the three plant types, *i.e.* their concentration in the reaction system was the highest in *flc*, intermediate in flc_{ABA}, and the lowest in RR plants.

Decarboxylation of [1-^{14}C]IAA. About two-thirds of the [1-^{14}C]IAA disappeared from the culture medium during the 24 h of the experiment (Table III). Auxin decarboxylation was similar in leaf discs of *flc* and RR plants. Somewhat higher proportion of the [1-^{14}C]IAA was decarboxylated in flc_{ABA} leaf discs.

Table I. Formation of Glu and IPyA by enzyme preparation extracted from shoots of mature RR, flc and flc_{ABA} plants

Each value is an average of 2 replications. The data represent the results of one out of 4 similar experiments.

Plant type	Glutamic acid	Indolepyruvic acid
	(µg/h .mg protein)	
RR	6.6	7.7
flc	14.8	12.3
flc_{ABA}	8.2	9.4

Ethylene Evolution. Mutant plants released more ethylene than the normal ones under both normal and high humidities (Table IV). ABA treatment greatly induced ethylene evolution in *flc*$_{ABA}$ plants (which resembled phenotypically the normal genotype), bringing it to the normal level or even lower. ABA-treated RR plants were similarly affected, although to a lesser extent. Ethylene evolution decreased in young *flc* plants and in older plants of both types under high humidity. Contrary to expectations, under high humidity, normal and mutant plants developed pronounced half epinasty and their apical dominance was very weak, similar to *flc* under normal and high humidity.

DISCUSSION

The excess of auxin-like substances in the mutant (13) can be explained, at least partly, by the higher activity of Trp-aminotransferase, which is the first enzyme in the pathway leading from Trp to IAA (10). The higher activity of this enzyme seems to be closely related to the reduced ABA level in this plant, as it was found to decrease in ABA-treated plants. The decomposition of exogenously supplied auxin was found (Table III) to be similar in *flc* and RR plants, which is in agreement with Tal *et al.* (15), who reported a similar IAA-oxidase activity in these plants. The present

Table II. Content of indole compounds of Trp-transamination produced by enzyme preparations extracted from mature RR, flc and flc$_{ABA}$ plants

Each value is an average of 2 replications. The data represent the results of one out of 2 similar experiments. Each spot corresponds to 2.5 mg protein.

Plant type	? R_f 0.00	? R_f 0.21	IPyA R_f 0.37	IAA R_f 0.60	IAAld R_f 0.73
			(Peak area, cm^2)		
RR	16.7	18.5	13.2	26.4	6.0
flc	52.2	54.5	36.0	35.2	17.6
flc$_{ABA}$	22.0	22.5	32.8	26.4	7.0

Table III. Decarboxylation of IAA-1-[^{14}C] by leaf discs of mature RR, flc and flc$_{ABA}$ plants

Total dpm about 29,000. Each value is an average of 10 replications.

Plant type	% of total dpm recovered during 24 h ± SE			
	In medium	In CO_2	In tissue	Total dpm recovered %
RR	34.6 ± 1.9	40.1 ± 3.5	25.3 ± 1.8	81.2 ± 1.3
flc	39.8 ± 3.2	39.5 ± 1.5	20.7 ± 1.6	79.0 ± 0.8
flc$_{ABA}$	30.8 ± 3.9	47.1 ± 3.1	22.1 ± 1.1	80.1 ± 1.9
Control	95.1 ± 1.7	4.9 ± 0.7		

Table IV. Ethylene evolution from shoots of young and mature control, ABA- and high humidity-treated RR and flc plants

Each value is an average of 5-6 replications.

Plant type	Treatments		
	Control	ABA[1]	High humidity[2]
		(µl ethylene/100 g dry wt ± SE	
RR young	26.5 ± 3.0	19.8 ± 2.2	22.3 ± 2.0
mature	82.7 ± 17.1	70.8 ± 15.8	54.7 ± 11.2
flc young	58.0 ± 7.9	17.1 ± 2.6	24.7 ± 1.7
mature	132.3 ± 20.5	70.8 ± 4.1	100.3 ± 16.6

[1]Young and mature plants were sprayed with ABA for 27 and 56 days, respectively.

[2]Young and mature plants were grown under high humitidy for 19 and 48 days, respectively.

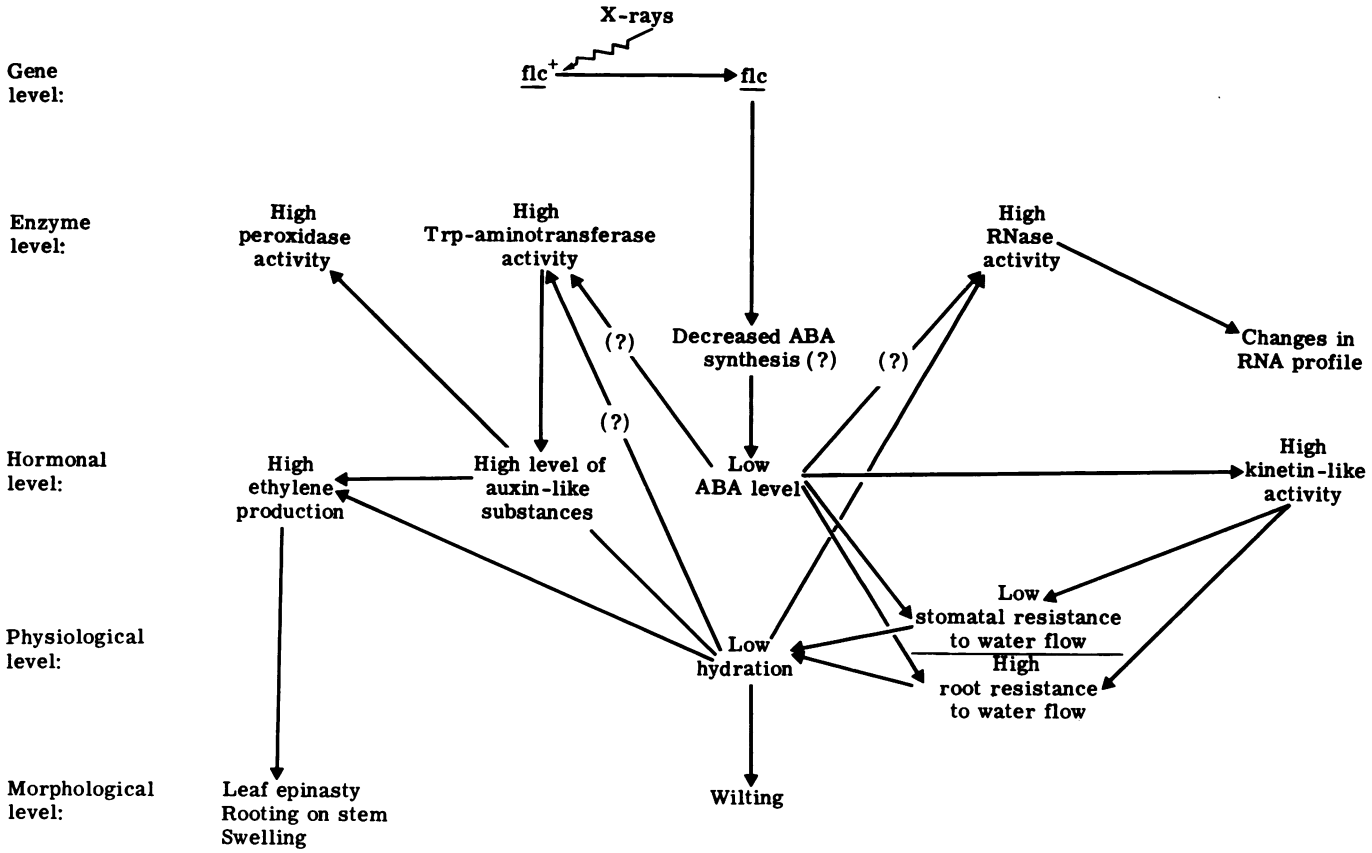

FIG. 1. Possible epigenetic sequence of gene action in *flacca*, a wilty mutant of tomato.

results suggest that the excess of auxin-like substances in *flc* is not caused by the reduction of auxin breakdown but by its enhanced synthesis.

Higher level of ethylene evolution was found (Table IV) in *flc* plants, as was expected in view of the larger amount of auxin-like substances present in *flc*, as compared with that in the normal RR plants (13). This finding is in agreement with the results of experiments in many other plant species, which showed that increased levels of auxin in the plant tend to stimulate ethylene production (1). ABA effected a reduction of ethylene evolution in flc_{ABA} plants, which may be explained, at least partly, by ABA effect on the level of auxin.

Tal (12) reported the appearance of symptoms characteristic of auxin excess in the developing *flc* plants. These symptoms include leaf epinasty, swelling of the upper stem, rooting along the stem, and pronounced apical dominance. The most prominent morphological symptom of *flc* plants in the present experiment was leaf epinasty. It is possible that at least the first three of these symptoms might be induced by the excess of auxin-like substances in the mutant, with ethylene acting as a mediator hormone, as suggested in other plants (1). Tucker (18) suggested that auxin may affect apical dominance in tomato in two ways: (*a*) by controlling the transport of cytokinins to the buds; and (*b*) by inducing the formation of ABA, which inhibits bud growth. At least the second mode of action does not seem to apply to the tomato mutant, in which ABA level is much lower than in the normal plants (13, 17).

It could be asked whether the greater ethylene evolution from the mutant plants, as compared with the normal plants, may result from a greater diffusion of the gas due to the excessive opening of stomata (which constitutes the major diffusion pathway) rather than from a greater production of ethylene.

The fact that ethylene evolution from young mutant plants growing under high humidity decreased to the normal level without a concomitant stomatal closure (verified by microscopical observations) suggests that it is not the greater diffusion through the stomata which is responsible for the higher ethylene evolution in the mutant but a greater production. The reduction of ethylene in the mutant under high humidity suggests that the water deficit in this plant plays an important role in ethylene production. Increase of ethylene production in plants subjected to water stress was reported in various species (3, 4, 8, 22). However, in mature mutant plants growing under high humidity, ethylene evolution decreased, but not as low as the normal level. This fact suggests that water deficit is not the only cause for the higher ethylene evolution in the mutant. It may be thus concluded that ABA affects ethylene production in the mutant partially through its effect on water balance and partially through its effect on auxin level.

Contrary to expectations, *flc* plants grown under high humidity, in which ethylene evolution decreased, developed more pronounced leaf epinasty than mutant plants grown under normal humidity. This phenomenon, which is impossible to explain at the present stage, only suggests that ethylene is not the only hormone which controls epinasty.

A scheme which describes the possible epigenetic sequence of gene action in the *flc* mutant and, at least partly, in two other wilty tomato mutants, *sit* and *not*, was suggested by Tal and Nevo (17). An up-to-date scheme is presented here (Fig. 1), which is based on findings of the present paper and the previous ones (7, 9, 12–17).

Acknowledgment—The authors wish to thank Mrs. Yona Beer for her skillful technical assistance.

LITERATURE CITED

1. ABELES FB 1973 Ethylene in Plant Biology. Academic Press, New York
2. ATFIELD GN, CJOR MORRIS 1961 Analytical separations by high voltage paper electrophoresis. Amino acids in protein hydrolysates. Biochem J 81: 606–614

3. BEN-YEHOSHUA S, B ALONI 1974 Effects of water stress on ethylene production by detached leaves of Valencia orange *Citrus sinensis* osbeck. Plant Physiol 53: 863–865
4. EL-BELTAGY AS, MA HALL 1974 Effect of water stress upon endogenous C_2H_4 levels in *Vicia faba*. New Phytol 73: 47–53
5. EPSTEIN E, S LAVEE 1975 Uptake and fate of IAA in apple callus tissue using IAA-1-^{14}C. Plant Cell Physiol 16: 553–561
6. HOAGLAND DR, DI ARNON 1950 The water-culture method for growing plants without soil. Calif Agric Exp Sta Univ Calif Berkeley, Circ 347
7. IMBER D, M TAL 1970 Phenotypic reversion of *flacca*, a wilty mutant of tomato, by abscisic acid. Science 169: 592–593
8. MCMICHAEL BL, WR JORDAN, RD POWELL 1972 Effect of water stress on ethylene production by intact cotton petioles. Plant Physiol 49: 658–662
9. PURI Y, M TAL 1977 Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. IV. Effect of abscisic acid and water content on RNase activity and RNA. Plant Physiol 59: 173–177
10. SCHNEIDER EA, F WIGHTMAN 1974 Metabolism of auxin in higher plants. Annu Rev Plant Physiol 25: 487–513
11. STUBBE H 1957 Mutanten der Kulturtomate, *Lycopersicon esculentum*, Miller I. Kulturpflanze 5: 190–220
12. TAL M 1966 Abnormal stomatal behavior in wilty mutants of tomato. Plant Physiol 41: 1387–1391
13. TAL M, D IMBER 1970 Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. II. Auxin- and abscisic acid-like activity. Plant Physiol 46: 373–376
14. TAL M, D IMBER 1971 Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. III. Hormonal effects on the water status in the plant. Plant Physiol 47: 849–850
15. TAL M, D IMBER, I GARDI 1974 Abnormal stomatal behaviour and hormonal imbalance in *flacca*, a wilty mutant of tomato. Effects of abscisic acid and auxin on stomatal behaviour and peroxidase activity. J Exp Bot 25: 51–60
16. TAL M, D IMBER, C ITAI 1970 Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. I. Root effect and kinetin-like activity. Plant Physiol 46: 367–372
17. TAL M, Y NEVO 1973 Abnormal stomatal behavior and root resistance, and hormonal imbalance in three wilty mutants of tomato. Biochem Genet 8: 291–300
18. TUCKER DJ 1977 Apical dominance in the "Rogue" tomato. Ann Bot 41: 181–190
19. WIGHTMAN F 1964 Pathways of tryptophan metabolism in tomato plants. *In* Regulateurs Naturels de la Croissance Vegetale. CNRS, Paris, pp 292–312
20. WIGHTMAN F, D COHEN 1968 Intermediary steps in the enzymatic conversion of tryptophan to IAA in cell-free systems from mung bean seedlings. *In* F Wightman, G Setterfield, eds, Biochemistry and Physiology of Plant and Growth Substances. Runge Press, Ottawa, pp 273–288
21. WIGHTMAN F, RA GIBSON, EA SCHNEIDER 1970 Evidence for the occurrence, formation and intermediary role of tryptamine in the biosynthesis of auxin in higher plants. Seventh Conference on Plant Growth Substances, Canberra, Australia, p 106 (abstract)
22. WRIGHT STC 1977 The relationship between leaf water potential (ψ leaf) and the levels of abscisic acid and ethylene in excised wheat leaves. Planta 134: 183–189