Stomatal Responses of Pearl Millet (*Pennisetum americanum* (L.) Leeke) Genotypes, in Relation to Abscisic Acid and Water Stress

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ABSTRACT

Stomatal responses to water stress and to applied ($\pm$)-abscisic acid (ABA) were examined in genotypes of pearl millet (*Pennisetum americanum* (L.) Leeke) known to differ in amounts of endogenous ABA accumulating during drought. In both a pot and a field experiment, Serere 39, a genotype with a high capacity to accumulate ABA, showed a higher stomatal sensitivity to water stress than did the 'low' ABA accumulator, BJ 104. In the field experiment, a third genotype, B282, accumulating least amounts of ABA, also had the lowest stomatal sensitivity to water stress.

There were no significant differences between these genotypes in stomatal response to applied ($\pm$)-ABA, or in the relationships between leaf conductance and levels of endogenous ABA. It is concluded that the differences in accumulation of endogenous ABA by these genotypes of pearl millet are of functional significance, and that endogenous ABA generated during a water stress which develops over days or weeks mediates stomatal responses to such stress.

INTRODUCTION

The substantial increase in the content of abscisic acid (ABA) in leaves of higher plants during periods of water deficit (Wright, 1978; Walton, 1980), is generally considered to have functional significance, being an adaptive response to water stress (Mansfield, Wellburn, and Moreira, 1978; Aspinall, 1980). A major role postulated for ABA during water stress is the mediation of stomatal closure (Mittelheuser and Van Steveninck, 1969; Jones and Mansfield, 1972); resulting in a reduction in water loss and in an improvement in water use efficiency (Jones and Mansfield, 1972; Mizrahi, Scherings, Malis Arad, and Richmond, 1974; Raschke, 1974; Dubbe, Farquhar, and Raschke, 1978).

Although exogenous ABA is an effective anti-transpirant (Jones and Mansfield, 1972; Davies, Mansfield, and Orton, 1978), its potential value as an aid to improving efficiency of water use is restricted, due largely to its lack of persistence in the plant. In view of this problem, and that of economic cost, a more satisfactory

alternative to hormone application is to develop varieties of crop plants which have an enhanced capacity to accumulate endogenous ABA. For several crop species, viz. maize (Larqué-Saavedra and Wain, 1974; 1976), sorghum (Larqué-Saavedra and Wain, 1976), wheat (Quarrie, 1978a, 1980; Quarrie and Jones, 1979), cotton (Ibragimov, Igamberdyeva, and Saidova, 1978), soybean (Samet, Sinclair, and Cortes, 1980), potato (Quarrie, 1981), and rice (Henson, unpublished results), intraspecific differences have been observed in the accumulation of ABA in response to water stress. In tomato and potato, strains have been isolated which do not accumulate significant amounts of ABA, and whose stomata remain open when under water stress (Tal and Imber, 1970; Tal and Nevo, 1973; Quarrie, 1981). In the other crop plants, in which intraspecific differences in ABA accumulation, thus far detected, were much less marked, few observations on the physiological consequences of the observed differences appear to have been reported. However, associations between ABA accumulation and drought resistance have been postulated (Larqué-Saavedra and Wain, 1976; Quarrie, 1978a; Ibragimov, 1978). The work reported in the present paper assessed the stomatal response to water stress of three genotypes of pearl millet (*Pennisetum americanum* (L.) Leeke), BJ 104, B282, and Serere 39. These had previously been found to differ, both in laboratory tests and in the field, in the amounts of ABA accumulating in the leaves during water stress (Henson, Mahalakshmi, Bidinger, and Alagarswamy, 1981a). In the present work stomatal responses to water stress of the genotypes have been related both to endogenous levels of (+)-ABA formed during stress, and to the effects on stomata of (±)-ABA applied to well-watered plants.

MATERIALS AND METHODS

Pot experiments

Plant culture

Seeds of pearl millet were sown, 2 per pot, in 9·0 cm diameter plastic pots containing *c* 400 g John Innes No. 2 potting compost. Seedlings were reduced to one per pot at the 2-leaf stage. Plants were grown in a glasshouse at Cambridge during the months of June to early September. Natural lighting was provided with night and day temperatures between 21 °C minimum and 34 °C maximum. Except when drought was being imposed, plants were continuously watered by means of a water-conducting wick threaded through the base of each pot.

Drought experiment

This compared the genotypes BJ 104 and Serere 39. Thirteen days after sowing, plants were selected for uniformity and allocated to treatments, allowing 6 replicate plants per treatment per harvest date. Plants to be water-stressed then received no further water while control plants continued to be watered by the wick system. The soil surface of droughted pots was covered to restrict water loss by direct evaporation. At the start of drought treatment (day 1) the lamina of the 5th leaf of BJ 104 was half emerged, while the 4th leaf was at a similar stage in Serere 39. Despite this difference in leaf emergence, dry weights of both shoot and roots at the start of the drought treatment were significantly ($P < 0.01$) greater for Serere 39 than for BJ 104.

Beginning on day 1 the two most recently fully expanded leaves (leaves 3, 4, or 5) on the main stem were sampled daily over 6 d between 11.00 and 13.00 h for stomatal conductance (g_L), leaf water potential (ψ), and ABA content. Conductance was determined for both leaf surfaces at adjacent positions near the mid-point, using a continuous flow diffusion porometer of a design similar to that of Day (1977). The lamina was then excised at its base and inserted rapidly into a pressure chamber. The pressure required to cause sap to rise to the cut surface was taken as a measure of ψ. In sampling for ABA content, the leaf was weighed after ψ determination, cut into 2–3 mm wide strips, and rapidly frozen by immersion in liquid nitrogen, prior to storage at below −20 °C, and analysis for ABA using the method of Quarrie (1978b).

Transpiration rates were determined concurrently using a separate but otherwise identical group of plants to those used for the above measurements. Pots were enclosed in polythene bags to restrict direct evaporation, and weighed twice daily to determine water loss. While one group was left to become droughted, control plants had water added after each weighing, to replace that lost by transpiration. Representative plants were sampled for dry weight at the beginning and end of the experiment.

Effects of applied ABA

Racemic *cis, trans*-abscisic acid (($\pm$)-ABA), obtained from Sigma Chemical Co. Ltd., was dissolved in $1 \cdot 0$ M NH_4OH, neutralized with acetic acid, and subsequently diluted with distilled water to give the required concentration. Control solutions, with ABA omitted, were similarly prepared.

Effects of ABA on conductance were assessed in two ways. In the first, whole plants were treated in the glasshouse by injecting $5 \cdot 0$ μl of a test solution of 1 μg μl^{-1} ABA into the main stem just above soil level. Plants were treated when leaf 4 was fully expanded, and the 5th leaf blade was approximately one third to half emerged. There were 10 replicate plants per treatment. About 3 h after injection, g_L of leaf 4 was determined as described above. Preliminary experiments established that ABA injected in the stem had maximum effect on g_L at this time. Injection of the control solution had not effect on g_L, as shown by comparison with untreated control plants.

In the second method, the effect on transpiration rate of imbibing detached leaves in ABA solutions was determined. When the 5th leaf blade was almost fully emerged, plants were transferred to a growth cabinet operated to provide a 12 h photoperiod, a 24 °C night and a 26 °C day temperature, with relative humidity maintained at 76%. Light, at 110 W m^{-2}, was provided by a combination of fluorescent and incandescent lamps, the latter providing 25% of the total irradiance. After 2 d the 5th leaf was detached in the dark about $2 \cdot 0$ cm above the ligule, and placed in a small glass vial containing 12 ml of test solution, the surface of which was covered to restrict evaporation. Lights were then switched on, and, after a stabilization period of 1 h, transpiration was monitored gravimetrically over the following 2 h. During this period, loss of leaf weight was found to be linear with time indicating that conductance was constant. At the end of the experiment leaf fresh weights and areas were determined, the latter by means of a closed-circuit television monitor and area quantifier. Six replicate leaves were used for each treatment.

Field experiment

The experiment was conducted at ICRISAT Center, near Hyderabad, India, in the dry season (January to April) of 1980. Three cultivars (BJ 104, Serere 39 and B282) were grown with or without regular irrigation. Irrigated plots received furrow irrigation at weekly intervals, while non-irrigated plots received their last irrigation 14 d after sowing (DAS). The experiment was laid out as a split-plot design with three replications; irrigation treatments formed main plots, split for genotypes.

Uppermost, fully expanded leaves (two per plot) were sampled between 11.30 and 12.30 h Indian Standard Time for g_L, ψ, osmotic potential (ψ_s) and ABA at approximately weekly intervals during the growing season. Samples were taken for ψ_s to allow the estimation of leaf turgor (ψ_p). Details of the methods used are given by Henson *et al.* (1981*a, b*). At the time of sampling, air temperatures were between 31 and 36 °C, relative humidity was generally below 40%, and irradiance above the canopy was about 700 W m^{-2}.

RESULTS

Stomatal response to water stress, and endogenous ABA; (i) pot experiment

A reduction in leaf ψ and transpiration rate of droughted plants became evident only after day 3 (Fig. 1). From day 4 there was a consistent and nearly linear decline in ψ with time. Leaf ψ of watered controls remained high and stable throughout the experiment. Serere 39 generally had a higher transpiration rate per plant than BJ 104 (Fig. 1c, F). This was mainly attributable to the larger size of Serere 39, as mean control conductances (g_L) of both genotypes were generally similar (Fig. 1B, E), except on day 3 when poor light conditions led to a lower g_L in
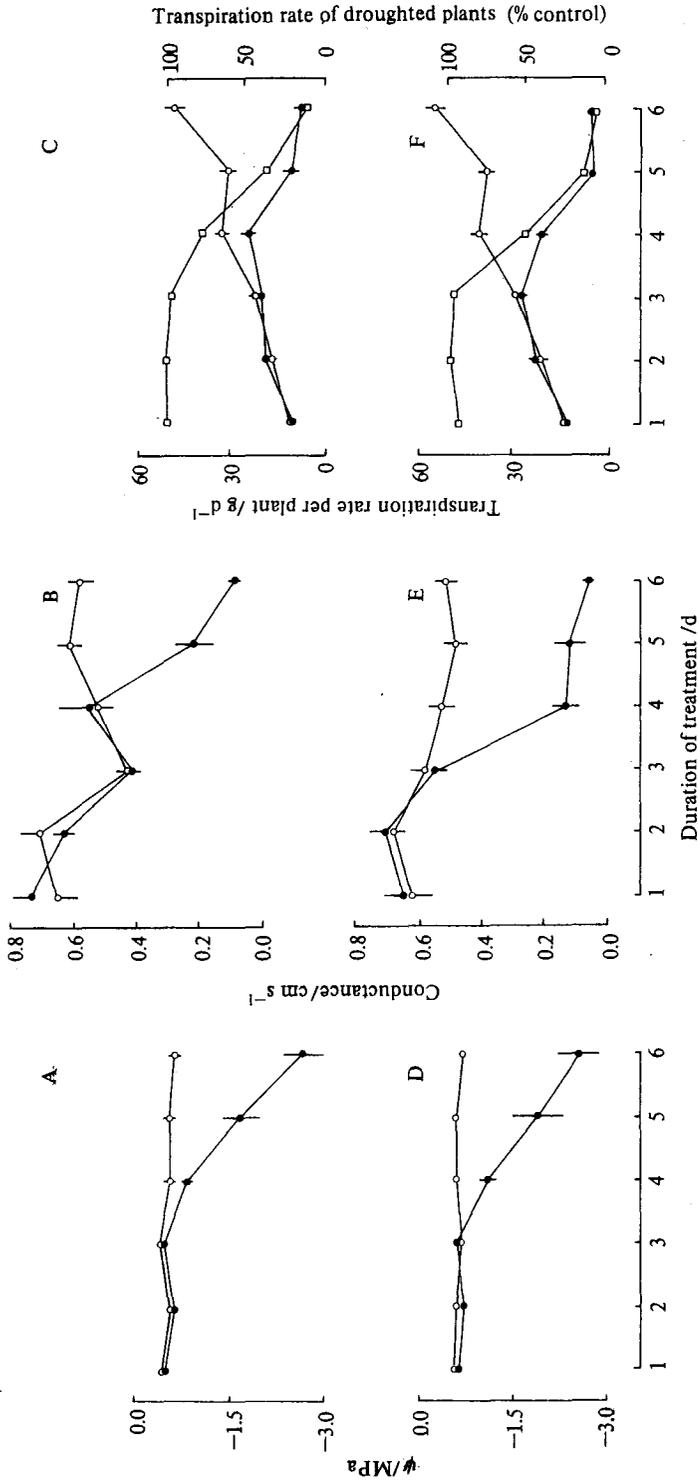
FIG. 1. Daily changes in leaf ψ (A, D), g_L (B, E), and whole plant transpiration rate (C, F), of watered (O) and droughted (●) plants of BJ 104 (A, B, C) and Serere 39 (D, E, F) grown in pots. Water was withheld from droughted plants on day 1. The transpiration rate of droughted plants as a percentage of watered plants (□) is also shown. Vertical bars indicate ± s.e.mean; for ψ and g_L, $n = 6$; for transpiration rate, $n = 10$.

BJ 104 than in Serere 39. Consequently, in Serere 39 there was an earlier depletion of soil water and hence an earlier onset of plant stress and decline in g_L. However, the relationship between leaf ψ and soil water content, assessed gravimetrically, did not differ between the two genotypes (results not presented), and mean rates of decline in ψ from day 4 to day 6 were similar for both genotypes ($0 \cdot 64$ and $0 \cdot 76$ MPa d^{-1} for Serere 39 and BJ 104 respectively).

As leaf ψ declined, g_L decreased more abruptly in Serere 39 than in BJ 104 (Fig. 2A, D). While stomatal closure occurred gradually in BJ 104 over a ψ range of about $0 \cdot 9$ MPa (from about $-0 \cdot 7$ to $-1 \cdot 6$ MPa), in Serere 39 closure commenced at about $-0 \cdot 7$ MPa and was essentially complete at $-1 \cdot 1$ MPa. Within genotypes, stomata of both leaf surfaces responded similarly, although abaxial g_L was initially higher than the corresponding adaxial values.

Increases in leaf ABA content occurred in both genotypes as leaf ψ declined below control values ($\simeq -0 \cdot 7$ MPa) (Fig. 2B, E). There was initially an approximately linear increase in ABA as ψ declined, but below a certain ψ ABA contents levelled off. During the linear phase the increase in ABA per unit reduction in ψ was estimated to be about 450 and 350 ng ABA g^{-1} fr. wt. MPa^{-1} for Serere 39 and BJ 104 respectively. The mean 'plateau' levels of ABA (340 ng g^{-1} fr. wt. for BJ 104; 372 ng for Serere 39) were similar for both genotypes. The ψ range over which the increase in ABA occurred ($\simeq -0 \cdot 75$ to $\simeq -1 \cdot 4$ MPa for Serere 39, $\simeq -0 \cdot 75$ to $\simeq -1 \cdot 7$ MPa for BJ 104) covered the ψ ranges over which stomata closed in the two genotypes.

There was no genotypic difference in the relationship between g_L and endogenous ABA content (Fig. 2C, F) suggesting that stomata of both genotypes were equally responsive to ABA. Conductance was linearly related to the log of ABA concentration ($P < 0 \cdot 01$).

When assessed over the first 7 d of withholding water, the drought treatment significantly ($P < 0 \cdot 01$) increased water use efficiency (WUE) from $5 \cdot 11$ to $5 \cdot 42$ mg dry matter increment g^{-1} H$_2$O (meaned over both genotypes). However, the interaction between genotype and treatment was not significant. Thus, increased ABA accumulation by Serere 39 at low ψ did not lead to any greater increase in WUE under drought by this genotype, compared with BJ 104.

Stomatal response to water stress, and endogenous ABA; (ii) field experiment

Stress developed slowly and at very similar rates for all three genotypes. Leaf ψ of droughted plants fell, over a 35 d period, from an initial value of $-0 \cdot 46$ MPa 24 d after sowing (DAS), by $0 \cdot 032$, $0 \cdot 030$, and $0 \cdot 029$ MPa d^{-1} for Serere 39, B282, and BJ 104 respectively.

As stress developed, g_L of droughted plants declined below that of controls (Fig. 3A, C, E), but treatment differences became significant only for Serere 39 ($P < 0 \cdot 01$; 52 and 59 DAS). Although g_L of irrigated Serere 39 plants had declined by the last two sampling occasions (in association with a decline in leaf ψ and an increase in ABA, Fig. 3B, D, F), g_L of droughted plants was nevertheless only 50 and 53% of control g_L on these two occasions, compared with corresponding values for BJ 104 of 75 and 80%. At these times absolute g_L values of droughted
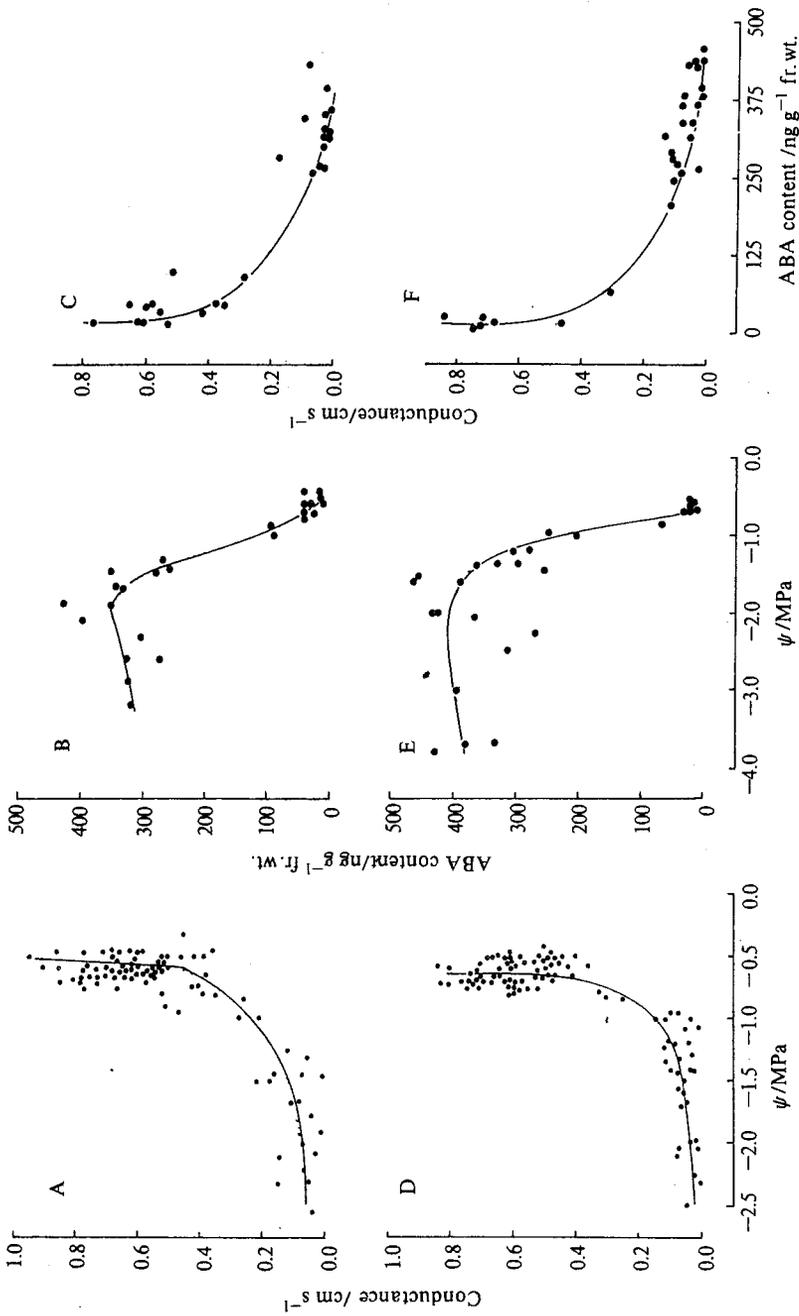
FIG. 2. Relationships between g_L and ψ (A, D), ABA content and ψ (B, E), and g_L and ABA content (C, F) for plants of BJ 104 (A, B, C) and Serere 39 (D, E, F) grown in pots. Each point represents a single leaf. Curves were fitted by eye.

Serere 39 plants were significantly ($P < 0.05$) less than those of other genotypes, being for example only 46 and 62% of BJ 104 values. These differences between genotypes remained unaffected when allowance was made for differences in ψ or ψ_p by covariance analysis.

Linear regressions of g_L on ψ, ψ_p, and ABA content were computed using data for the last three harvest occasions when differences between treatments and genotypes in g_L and ABA content became evident (Fig. 3). There were significant ($P < 0.05$–0.001) correlations between g_L and the other variables and some significant differences in regression coefficients for g_L and ψ, and g_L and ψ_p, between genotypes (Table 1). Stomata closed to a significantly ($P < 0.05$) greater extent per unit decline in ψ or ψ_p in Serere 39 than in B282, while stomatal sensitivity of BJ 104 to the development of stress was intermediate between that of the other two genotypes, and did not differ significantly from either.

In contrast to the genotype differences in g_L at equivalent ψ or ψ_p, the relationship between g_L and endogenous ABA content (Table 1) was very similar for the three genotypes.
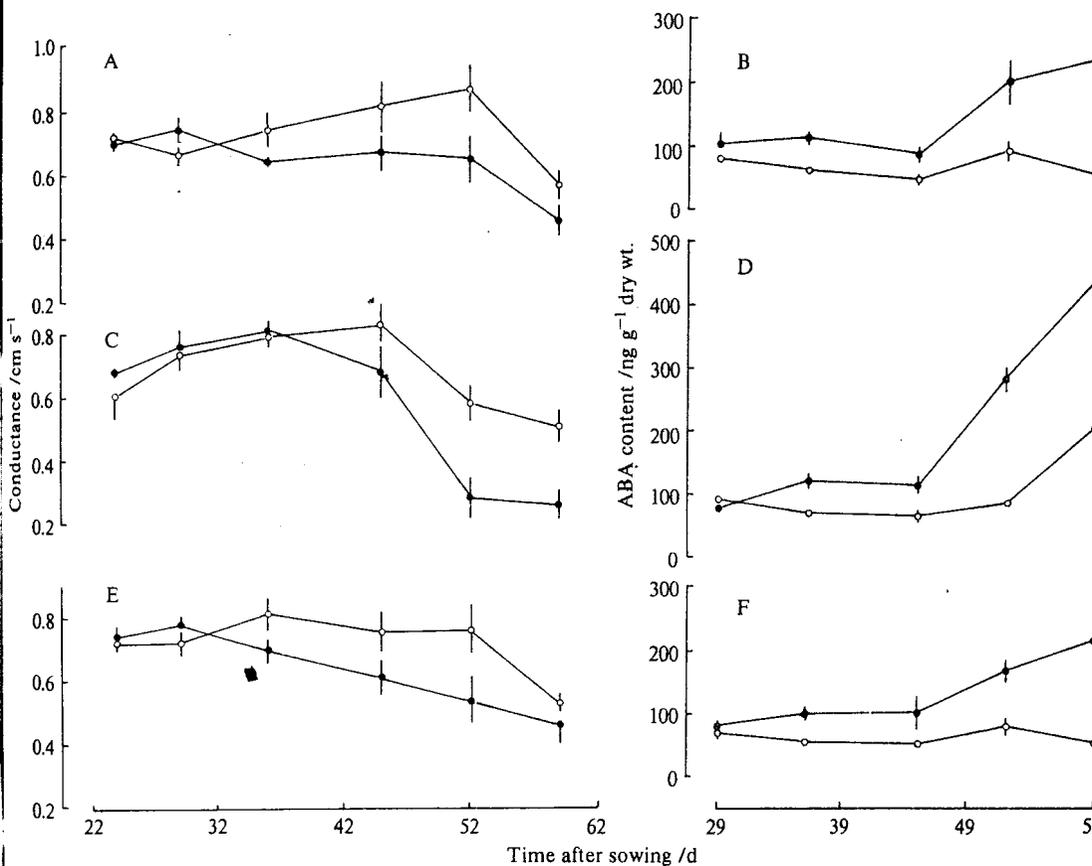

FIG. 3. Seasonal changes in g_L (A, C, E) and ABA content (B, D, F) of leaves at mid-day of plants of BJ 104 (A, B), Serere 39 (C, D), and B282 (E, F) grown with (O) or without (●) irrigation in the field. Vertical bars indicate $\pm$ s.e.mean; $n = 6$.

TABLE 1. *Correlation (r) and linear regression (β) coefficients relating* g_L *to* Ψ, Ψ_p, *and ABA content of three genotypes of pearl millet grown in the field*

Data refer to samples of pearl millet leaves taken 45, 52, and 59 d after sowing. Statistical significance at $P < 0.05$, <0.01, and <0.001 is indicated by *, **, and *** respectively; d.f. = 34.

	Genotype	r	$\beta \pm$ s.e.
g_L v. Ψ	BJ 104	0·49**	0·023 ± 0·007
	Serere 39	0·71***	0·042 ± 0·007
	B282	0·46**	0·019 ± 0·006
g_L v. Ψ_p	BJ 104	0·42*	0·032 ± 0·012
	Serere 39	0·59***	0·052 ± 0·012
	B282	0·37*	0·022 ± 0·009
g_L v. ABA	BJ 104	−0·57***	−0·0012 ± 0·0003
	Serere 39	−0·76***	−0·0013 ± 0·0002
	B282	−0·45**	−0·0010 ± 0·0003

Stomatal response to exogenous (±)—ABA

Preliminary experiments with BJ 104 established that 5 μg (±)—ABA per plant, injected into the shoot base as a 5 μl aqueous solution, only reduced g_L by about 20% while markedly inhibiting leaf extension. Effects on g_L were most pronounced about 2–3 h after application and, while there was still a noticeable effect after 24 h, by 48 h g_L had recovered to levels found in untreated plants.

Using the injection method no significant differences were found between BJ 104, Serere 39, B282 (or between these and 13 other genotypes tested) in the effect of ABA on g_L measured 2–3 h after ABA application. Similarly, measuring transpiration rates of excised leaves imbibed in ABA solutions revealed no significant genotypic differences. Transpiration was inhibited to a similar extent in BJ 104 and Serere 39, with both genotypes showing a similar response to a range of ABA concentrations (Fig. 4). Similar results were also obtained in comparisons of Serere 39 and B282 over the same range of ABA concentrations.

DISCUSSION

The main object of the present studies was to determine whether relatively small differences within a species in the accumulation of endogenous ABA during water stress were associated with differences in stomatal response. There are few examples where within-species variation in endogenous ABA concentration has been associated with variation in stomatal behaviour. 'Wilty' mutants of tomato (Tal and Imber, 1970) and potato (Quarrie, 1981) were found to be deficient in ABA (having only 10–30% of the ABA content of normal plants), and also incapable of closing stomata when water-stressed. However, in these ABA-deficient mutants stomata also fail to close appreciably in darkness, or when exposed to plasmolytic solutions (Tal, 1966; Imber and Tal, 1970; Tal, Imber, and Gardi, 1974; Quarrie, 1981). This abnormal stomatal behaviour complicates interpretation of the mechanisms in these mutants which result in failure of stomata to respond to water stress.
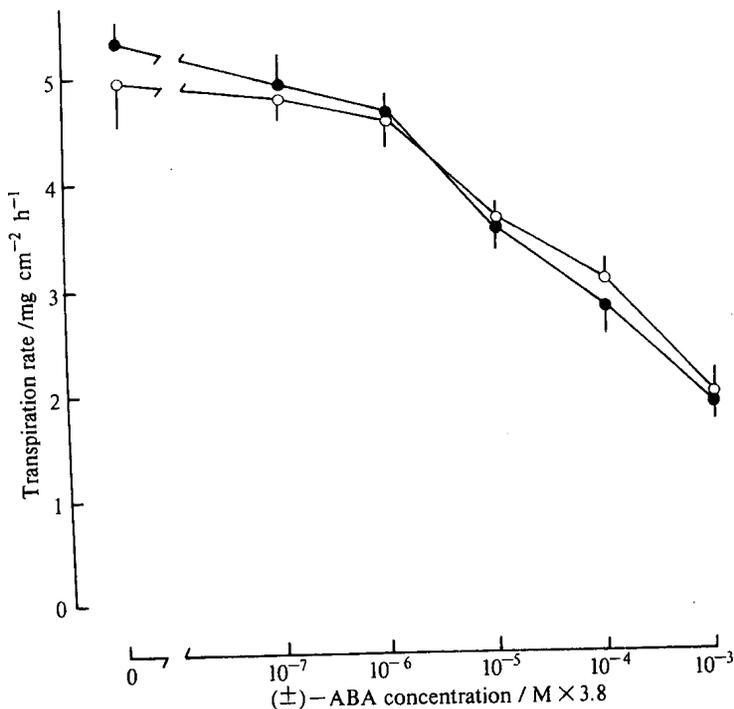
FIG. 4. The effect of $(\pm)$-ABA, supplied to detached leaves via the cut end, on the transpiration rate of BJ 104 (O) and Serere 39 (●). Vertical bars indicate $\pm$s.e.mean; $n = 6$.

In pearl millet smaller, but nevertheless significant, differences were detected between genotypes in amounts of ABA accumulating in response to water stress (Henson *et al.*, 1981*a*). These differences were evident under both laboratory and field conditions and did not result from variation between genotypes in leaf water content, ψ, or ψ_p. The results of the present study suggest that such genotypic differences in ABA accumulation have consequential effects on stomatal functioning during water stress. Thus, Serere 39, a variety with a high ABA-accumulating capacity (Henson *et al.*, 1981*a*) showed a greater degree of stomatal closure, both a response to relatively rapidly-induced stress in a pot experiment (Fig. 2), and to more slowly-induced stress in the field (Table 1), than the low accumulator BJ 104. Of the genotypes studied, B282 had the lowest ABA accumulation, and had the least sensitive stomatal response to water stress in the field.

Although, when rapidly stressed in pots, the differences between BJ 104 and Serere 39 in ABA accumulation were not as great as those detected in other tests (Henson *et al.*, 1981*a*), the associated differences in g_L (Fig. 2c, F) were exactly those expected on the assumption that stomata of the two genotypes were equally affected by a given amount of ABA accumulated during stress. This conclusion is supported by the relationships shown in Fig. 2c, and F and Table 1, and by the equal responsiveness of stomata of the two genotypes to exogenous $(\pm)$-ABA (Fig. 4). The differences in ABA content between BJ 104 and Serere 39 in the pot

experiment were most apparent at the relatively low stress levels ($\psi > -1.0$ MPa) at which stomatal closure was elicited. Differences in ABA content at high levels of stress were small, and would have been much less important in terms of their potential effect on stomatal closure, because closure was by then largely complete.

Under field conditions, stomatal closure occurred gradually over a much wider range of ψ than in the pot experiment. Such stomatal adaptation to the field environment is common and has been ascribed to osmotic adjustment (Ludlow, 1980). However, osmotic adjustment in millet was insufficient to maintain bulk leaf turgor (Henson *et al.*, 1981*b*), yet g_L remained appreciable, even at zero ψ_p. Changes in ABA with ψ were also more gradual in the field than in the pot experiment, and this may account for the different stomatal response patterns under the two conditions. Thus, ABA concentrations attained in field-grown plants were considerably lower, at equivalent ψ, than concentrations found in plants droughted rapidly in pots. From the regression of g_L on ABA (Table 1), a reduction in total g_L to 0.2 cm s^{-1} in the field occurred at a mean endogenous ABA content of 460–500 ng g^{-1} dry wt., equivalent to about 115–120 ng g^{-1} fr. wt. This compares closely with corresponding estimates for pot-grown plants (Fig. 2c, F) of 130–150 ng g^{-1} fr. wt. for a g_L of 0.2 cm s^{-1}. Thus, although environmental conditions, particularly the rate at which stress developed, differed markedly between pots and the field, stomatal conductance showed a roughly similar relationship to ABA content and, furthermore, differences between genotypes were similarly expressed in both situations.

The results of the present experiments, in which water stress developed either over days or weeks, indicate that ABA accumulating in the leaf during water stress acts to mediate stomatal closure. Differences between genotypes in the capacity to accumulate ABA were expressed as differences in stomatal response to water stress, and may therefore be significant in terms of influencing water use and survival under drought conditions. Although available results do not support the contention that high ABA accumulation necessarily leads to an improved water-use efficiency, it is possible that other physiological or morphological and anatomical differences between genotypes negated this benefit expected with high ABA accumulation and rapid stomatal closure. Further studies of this aspect, using closely related lines contrasting in ABA-accumulating capacity, are envisaged.

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