Jpn. J. Crop Sci. 63(4) : 676—681 (1994)

Effects of Epibrassinolide and Abscisic Acid on Sorghum Plants Growing under Soil Water Deficit

II. Physiological basis for drought resistance induced by exogenous epibrassinolide and abscisic acid*

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Received April 1, 1994

Abstract : It was reported that pretreatment with abscisic acid (ABA) increased the drought resistance of sorghum plants and that 22R, 23R epibrassinolide (EBR) enhanced the effects of ABA. To explore the mechanistic basis for drought resistance induced by ABA and/or EBR, the effects of the treatments on water retention were examined using the transpiration decline curve of excised leaves. The results are summarized as follows.

Spray treatment with ABA or EBR increased *in situ* relative water content ($RWC_{l.s}$). $RWC_{l.s}$ in ABA+EBR-treated plants was larger than in plants treated with ABA or EBR alone. This suggested that EBR enhanced the effect of ABA. Using the transpiration decline curve, we found that both ABA and EBR diminished the stomatal transpiration rate, but continued it at a lower RWC for a longer time. This effect of ABA+EBR was also larger than that of ABA or EBR alone. ABA treatment significantly decreased the cuticular transpiration rate. EBR did not show this effect by itself, but enhanced the effect of ABA. The effects of these treatments on glaucousness of the leaf surface coincided well with those on cuticular transpiration. This suggests that ABA promoted wax deposition on the leaf surface. Relative water content when stomata closed was in the order of Control>EBR>ABA>EBR+ABA. This suggests that treatment with EBR, ABA and their combination not only increased water retention but also enhanced physiological tolerance to low water status. Synergistic interaction between ABA and EBR was observed in all the instances examined. The increase in water maintaining capacity and enhanced tolerance accounted for the drought resistance reported in the previous paper.
Key words : Abscisic acid, Brassinolide, Cuticular transpiration, Drought resistance, Sorghum, Stomatal transpiration, Water stress.

エピブラシノライドおよびアブシジン酸が土壌水分欠乏下に生育するソルガムに及ぼす影響　第2報　葉面散布処理による耐乾燥性増強効果の発現機構：徐会連・志田篤彦・二谷文夫・玖村敦彦（日本化薬上尾研究所）

要　旨：アブシジン酸（ABA）とエピブラシノライド（EBR）の耐乾燥性増強効果の発現機構について，蒸散低下曲線から求めた気孔（TR_{sm}）・クチクラ（TR_{cc}）両蒸散速度およびそれらに関係する要素から検討した．1）EBR と ABA は TR_{sm} を減少させ，両者を混合する場合この減少程度はいっそう大きかった．2）ABA は TR_{cc} を減少させ，EBR は単独では TR_{cc} を減少させなかったが，ABA と併用すると，ABA の前記の効果を強めた．3）葉面灰色度（Gn）に対する各処理の効果は TR_{cc} に見られた傾向と完全に対応することから，上記薬剤による TR_{cc} の抑制は，ワックスの沈着促進を通じて惹起されたことが示唆された．4）切り葉を十分吸水させたうえ無給水で3時間蒸散させた後の相対含水率（RWC_{3h}）と水不足下の植物の着生葉の相対含水率（$RWC_{l.s}$）との傾向が一致していることから，TR_{sm} と TR_{cc} の抑制による水分損失の軽減が乾燥下で葉の水分を高く保つ原因の一部となっていると推察された．5）気孔が閉じる時の相対含水率（$RWC_{s.c}$）が対照>EBR>ABA>EBR+ABA であったことから，これらの処理により，水分損失を少なくするばかりでなく，葉内水分不足の場合にもより正常な機能を維持する能力を強めることが示唆された．以上から，個体あるいは器官のレベルで見られた耐乾燥性の生理的基礎の一部は，気孔蒸散，クチクラ蒸散による水分の損失の軽減と，組織内水分低下に対する生理的メカニズムの耐性の強化と考えられた．
キーワード：アブシジン酸，気孔蒸散，クチクラ，ソルガム，耐乾燥性，ブラシノライド，水ストレス．

* Part of this paper was presented at the 189th Meeting of the Japanese Society of Crop Science, April 3—4, 1990, Tokyo. This study was supported in part by the Association for Overseas Technical Scholarships.
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In our previous study[14] it was reported that the spray treatment of sorghum plants with abscisic acid (ABA) promoted growth and enhanced survival ability under soil water deficit, and that 22R, 23R epibrassinolide (EBR) strengthened the effect of ABA when applied in combination with ABA.

In the same paper[14], the basis for ABA and EBR to increase drought resistance was examined in terms of root growth. The results suggested that the increase in drought resistance might be, at least in part, attributed to promotion of root growth by the treatments, which might result in an increase of water uptake under limited soil water supply. However, the water status of a plant is determined not only by the water uptake of roots but also by water loss from aerial parts. Thus, in the present study, the authors examined the effects of ABA and EBR on leaf characteristics related to water loss. Furthermore, leaf tolerance to water deficit was also examined in terms of minimum water content needed to keep stomata open.

Materials and Methods

1. Plant materials

Seeds of grain sorghum (*Sorghum bicolor* (L.) Moench cv. Lucky) were sown in accordance with the methods used in the previous study[14]. Seeds were sown in 1/5000 a Wagner pots filled with loamy soil on June 22, 1989. Compound fertilizer ($N : P_2O_5 : K_2O = 16 : 14 : 12$) was applied at a rate of 2 g per pot as basal dressing. At three leaf stage of growth, seedlings were thinned out so that four plants with similar size might remain in each pot. Plants were grown in a natural-light greenhouse, where it was hot and dry (daily maximum temperature, ca 36°C; daily minimum air humidity, ca 40%).

The pots were irrigated to saturation on June 22, but thereafter supplied with no water. Spray treatments were conducted on the 4th, 7th, 11th and 14th day from the last saturated irrigation (June 22). Four solutions were used for the spray treatments: No. 1—EBR (Nippon Kayaku Co. Ltd.) 0.1 ppm, No. 2—ABA (Tokyo Kasei Industry Co. Ltd.) 50 ppm, No. 3—EBR 0.1 ppm+ABA 50 ppm, and No. 4—Water (control), respectively. "Sin-glamine" (Sankyo Co. Ltd.) was used as a wetting agent in all of the treatments including control. Four plants in the same pot were labeled as No. 1 to No. 4 and treated with the corresponding four solutions abovementioned, respectively. In this way, the effects of treatments were compared under exactly the same soil water regime. Five soil water regimes were designed as follows.

1) After saturated irrigation on June 29, plants were not supplied with water until the final sampling on July 18. However, the seedlings did not show any sign of wilting during this period.

2) Plants were not supplied with water until July 18 and then they were supplied with water and thereafter grew under sufficient water conditions until August 9.

3) After June 29, the plants were not supplied with any water until the end of the experiment (August 2).

4) After the first wilting (July 18), plants were not supplied with water for a period and then they were watered sufficiently until the end of the experiment (August 9).

5) After the first wilting (July 18), plants were not supplied with water for about three weeks. Consequently, plant growth was strongly depressed. After rewatering on August 8, the plants began to regrow gradually.

2. Measurement

(1) Relative water content *in situ* ($RWC_{l.s}$)

At midday on July 18, when about half the number of 6th leaf blades of all the plants appeared to wilt, 20 of the 6th leaves (the second fully-expanded leaf from the top without processes of cell enlargement and senescence) were excised from the stems and their fresh weight was immediately determined. Then the leaves were put into a 1-litre measuring cylinder containing some water, with the cut ends immersed in the water. The cylinder was covered with a piece of parafilm to keep the air humidity in it at 100%, and placed in the dark. After 16 hours, when the leaves had been fully rehydrated, they were drawn out of the cylinder and water adhering to the leaf surface was removed with blotting paper. Half the number of leaves were weighed to evaluate "Saturated weight" and then dried in an oven for measuring dry weight. Relative water content *in situ* ($RWC_{l.s}$) was calculated as

$RWC_{l.s} = 100 \times$ [(Fresh weight just after cutting) − (Dry weight)]/[Fresh weight after rehydration) − (Dry weight)].

(2) Stomatal transpiration rate (TR_{sm}) and cuticular transpiration rate (TR_{cc})

Another half of the rehydrated leaves was transferred into an environment-controlled chamber, where the air temperature, relative air humidity and light intensity were regulated at 25°C, 65%, and 400 μmol m^{-2} s^{-1} (400-700 nm), respectively. First, the leaves were irradiated with their cut ends immersed in water to make stomata open. Then the leaves were taken out of water and blotted to remove the adhering water. Their cut ends were smeared with lanolin to prevent water loss. The leaves treated as above were placed on a frame net on the dish of an electronic balance and the weight was recorded at 2 to 15-min intervals over a 3-hour period in the controlled chamber. The so-called transpiration decline curve[3,4,5,6,10,11,12)] was obtained by plotting the leaf RWC against time (Fig. 1).

The transpiration decline curve obtained as mentioned above exhibited two distinctive linear parts, i.e., the steep part on the left (L_1) and the gently sloped part on the right (L_2) as in the cases of cotton and barley as reported by Quisenberry et al[12)] and Cetl[3)]. According to Quisenberry et al.[12)], the slope of L_1 shows the rate of water loss mainly through stomatal transpiration and partly through cuticular transpiration, and the slope of L_2 practically represents cuticular transpiration. Therefore, in case L_1 and L_2 intersect the abscissa with angles of α and β, respectively, the rate of cuticular transpiration (TR_{cc}) was estimated as follows.

$$TR_{cc} = \tan \beta.$$

Since the slope of L_1 represents the sum of stomatal and cuticular transpiration, the rate of stomatal transpiration (TR_{sm}) was derived as follows.

$$TR_{sm} = \tan \alpha - \tan \beta.$$

(3) Relative water content when stomata closed ($RWC_{s.c}$)

In case L_1 and L_2 crossed with each other at point (t, w), "w" was defined as "relative water content when stomata closed" ($RWC_{s.c}$). $RWC_{s.c}$ will be used as an index to indicate the ability of stomata to stay open under leaf-water deficit conditions.

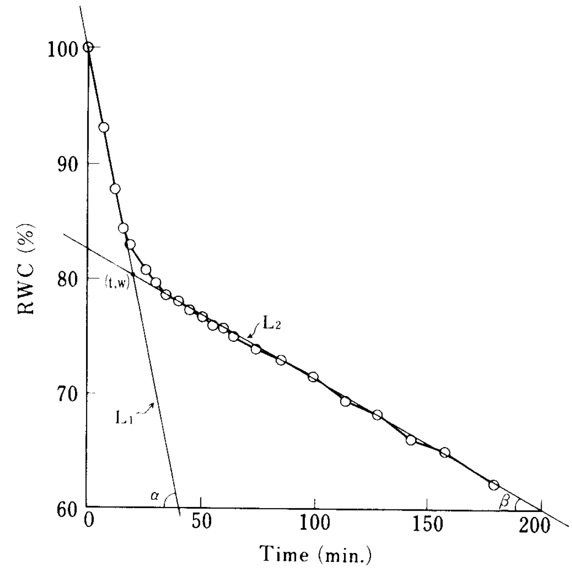

Fig. 1. An example of the transpiration decline curve of sorghum leaves.

(4) Period during which stomata remain open after water supply ceased ($PER_{s.o}$)

The above-described "t" is the time of stomatal closure and this indicates how long stomata are able to remain open after the water supply ceased.

(5) Relative water content after a 3-hour period (RWC_{3h})

After a 3-hour period of transpiration, the fresh and dry weight of the sample leaves were determined. The RWC derived in this manner was defined as "RWC after a 3-hour period" (RWC_{3h}) and used as an index of the integrated capacity for water retention.

(6) Glaucousness of leaf surface (Gn)

On July 20, when all the 6th leaves wilted, their "glaucousness" (Gn) was visually determined as levels 1 to 5. Level 1 means the minimum Gn and Level 5 the maximum. Glaucousness evaluated in such a way has been used as an index for degree of wax deposition on leaf surface[1,2,8,9,10,11,12,13)].

Results

The form of transpiration decline curve varied in response to treatments (Fig. 2). TR_{sm}, TR_{cc}, $PER_{s.o}$, $RWC_{s.c}$ and RWC_{3h} derived from the curves were presented in Table 1 together with $RWC_{i.s}$ and Gn obtained according to the above-mentioned procedure.

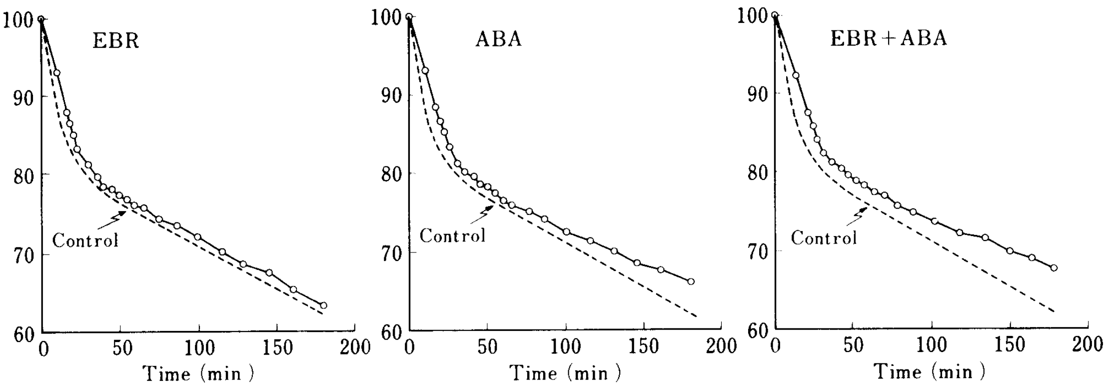

Fig. 2. Transpiration decline curves of the 6th leaf blades from EBR, ABA and EBR+ABA-treated sorghum plants.

Table 1 Effects of epibrassinolide (EBR) and abscisic acid (ABA) on factors related to the ability of water retention in sorghum plants.

Treatment	$RWC_{i.s}$ (%)	Gn	TR_{sm} (%TW h^{-1})*	TR_{cc}	$PER_{s.o}$ (min)	$RWC_{s.c}$ (%)	RWC_{3h}
Control	76.5^{a}	2.0^{A}	48.8	6.7	19.4	80.7	62.3
EBR	77.2^{a}	1.9^{Aa}	41.1	6.6	25.8	79.4	63.2
ABA	77.9^{a}	3.7^{ABb}	38.0	5.2	29.0	78.7	66.1
EBR+ABA	79.7^{b}	4.2^{Bc}	33.7	4.5	33.1	78.4	68.4

* This means percent of total tissue water lost in one hour. $RWC_{i.s}$, *in situ* relative water content ; Gn, the glauconsness of the leaf surface ; TR_{sm} and TR_{cc}, cuticular and stomatal transpiration rates, respectively ; $PER_{s.o}$, the time during which stomata are open ; $RWC_{s.c}$, relative water content at which stomata begin to close ; RWC_{3h}, relative water content after 3-hour period of transpiration. The superscript with different small letters means a significant difference at $P \leq 0.05$ level and that with different capital letters, at $P \leq 0.01$.

1. Relative water content *in situ* ($RWC_{i.s}$)

$RWC_{i.s}$ was increased by EBR and ABA treatment to a larger extent by the latter though the increases were statistically insignificant. The increase of $RWC_{i.s}$ by the EBR+ABA treatment was the largest of all and the difference between the control and this treatment was significant at the 5% level.

2. Relative water content after a 3-hour period of transpiration (RWC_{3h})

RWC_{3h} shows the integrated capacity for water retention induced by both diminished stomatal and cuticular transpiration. The order of RWC_{3h} was EBR+ABA>ABA>EBR>Control.

3. Stomatal transpiration (TR_{sm})

The treatment with EBR and that with ABA both decreased TR_{sm} considerably. The decreasing effect was a little larger in the latter. The extent of decrease by a treatment was the largest when these two substances were combined, i.e. in the treatment of EBR+ABA. Since measurement was not repeated, the significance test was not done in the results of TR_{sm}, TR_{cc} and PER_{so}.

4. Cuticular transpiration rate (TR_{cc})

ABA decreased TR_{cc} to some extent. EBR applied singly had little effect on TR_{cc} but that in combination with ABA strengthened the above-mentioned effect of ABA.

5. Relative water content when stomata closed ($RWC_{s.c}$)

$RWC_{s.c}$ was decreased by the treatments of EBR, ABA, and EBR+ABA. The extent of the decrease was in the order of EBR+ABA>ABA>EBR.

6. Reriod during which stomata remain open after water supply was stopped ($PER_{s.o}$)

$PER_{s.o}$ was increased considerably by all of the three treatments. The extent of the increase was in the order of EBR+ABA>

ABA > EBR

7. Glaucousness (Gn)

ABA significantly increased Gn level. Though EBR singly applied did not affect Gn level, that applied in combination with ABA strengthened the above-mentioned effect of the latter considerably.

Discussion

In the case of the experiment of transpiration decline using excised leaves, leaf water content was related with only stomatal and cuticular transpiration rates, since the water uptake was absolutely ceased. Under *in situ* conditions, leaf water content was related to water uptake by root as well as water loss by transpiration. It was reported in the previous paper that treatment with ABA and EBR+ABA promoted root growth, which might account for survival and growth under drought conditions. The present work was focused on leaf characteristics related to water loss.

RWC_{3h} obtained from the transpiration decline curve shows the integrated capacity for water maintenance induced by both diminished stomatal and cuticular transpiration. The value of RWC_{3h} was in the order of EBR+ABA > ABA > EBR > Control. This was attributed to both diminished stomatal and cuticular transpiration by the treatment, with the exception of no effect of EBR on cuticular transpiration. This is consistent with that of $RWC_{l.s}$. However, the maintenance of $RWC_{l.s}$ was not only attributed to the decrease of water loss but also to promoted water uptake by the developed root system[14].

Comparing the values of cuticular transpiration rates with those of glaucousness, we found that the larger the extent of glaucousness, the smaller the cuticular transpiration rate. It is well documented that glaucousness is positively correlated with the deposition of wax on and in the leaf[1,2,9,10,11,12,13]. Therefore, it can be suggested that decrease in cuticular transpiration by ABA or EBR+ABA was through promotion of wax deposition.

Wax deposition is suggested as a manifestation of acclimation of plants to water stress, which is inversely correlated with cuticular transpiration[5,8,10,11]. Cuticular transpiration decreases when cuticular wax is increased by environmental stresses[5,9]. Under severe drought conditions, stomata close and cuticular transpiration dominates plant water loss[8,9]. In such a case, cuticular wax becomes very important and may be a major factor in overall drought resistance via dehydration avoidance[8,9]. Therefore, it is reasonably suggested that the increase in survival by ABA or EBR+ABA treatment was attributed to the promotion of wax deposition and consequent decrease in water loss through cuticular transpiration. The extent of plant or leaf survival and the growth rate under severe drought conditions were in the order of EBR+ABA > ABA > EBR > Control[14], which coincided inversely with the cuticular transpiration rates.

Comparing the value of $RWC_{l.s}$ at midday, when the leave showed symptoms of wilting with the $RWC_{s.c}$ obtained from the transpiration decline curve, we found that the magnitude (in the range of 76% to 80%) was similar for the two cases as a whole. It is suggested that sorghum plants are able to grow and survive at the critical point of RWC when stomata close or begin to close. Comparing the values of $RWC_{l.s}$ and $RWC_{s.c}$ among control, EBR, ABA, and EBR+ABA-treated plants, the degree of $RWC_{l.s}$ over $RWC_{s.c}$ was larger for the latter than the former. This suggests that the chance for stomata to open and photosynthesis to take place was larger in the latter than in the former. This suggestion was supported by the results of growth under severe water stress, i.e., EBR+ABA > ABA > EBR > Control[14].

Under severe drought conditions, EBR had effects similar to ABA, with the exception of no effects on cuticular transpiration and glaucousness. This suggests that water maintenance induced by EBR treatment was mainly a physiological rather than a morphological consequence, but that the effect of ABA is the consequence of both physiological and morphological changes if the stomatal response and leaf surface characteristcs were considered as indicators of physiology and morphology, respectively. The results in all of the aspects examined suggest that EBR strengthened the effects of ABA on both physiological and morphological bases.

The so-called transpiration decline curve has been used to determine the water maintaining ability of plants[3,4,5,6,10,11,12]. Cetl[3] used the cuticular loss of water from detached leaves after hydroactive stomatal closure to test for drought resistance in barley. Quisenberry et al.[12] divided the transpiration decline curve into stomatal and cuticular phases and determined the time and relative water content at stomatal closure. They concluded that leaves of the cultivars developed in semi-arid environments reached a lower RWC before stomatal closure than leaves from the cultivars developed in well-watered environments. Since ABA has effects on plants similar to those of water stress treatment, the result in the present work is consistent with the abovementioned. Here, it must be pointed out that the meaning of the stomatal transpiration rate obtained from the transpiration decline curve is different from that measured *in situ* by the gas exchange method. Under soil water deficit conditions in the fields, plants having higher drought tolerance (not avoidance) or higher osmotic adjusting ability can maintain higher stomatal transpiration by larger stomatal opening in comparison with the drought sensitive plants[7]. The stomatal transpiration rate obtained from transpiration decline curve is usually smaller in drought tolerant plants, which can, however, maintain lower stomatal transpiration for a longer period[2].

Richards et al[13] suggested that glaucousness increases grain yield and dry matter of droughted wheat, primarily through effect on water use efficiency, on an extended duration of transpiration, and on an increase of leaf surviving duration. Our results in the present study about glaucousness, combined with results in the previous paper about drought resistance and survial, are consistent with the above-mentioned suggestion, although the epicuticular wax deposition was not directly determined. In conclusion, treatments with EBR, ABA, and their combination not only increased the ability of water retention but also enhanced physiological tolerance to low water status.

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