REV. CS. AGROPEC. VII: 13-17. Córdoba, 1990

# Endogenous growth inhibitors, nodulation and nitrogen fixation in soybean under drought and treated with gibberellic acid and abscisic acid

Racca R.W.; Argüello J.A.; Núñez S. B.; Luna V.; Frioni L. y Bottini R.

## SUMMARY

Soybean plants (*Glycine max* L. Merrill) that had reached full nodulation were submitted to physiological water stress by using polyethylen glycol (PEG 6000). Gibberellin $A_3$ and/or abscisic acid were sprayed in an attempt to alleviate or mimic the stress. After rewatering the plants, endogenous growth inhibitors in the root system, nitrogenase activity (as per root or nodule basis) number of nodules per plant, and the fresh and dry weight of hodules and roots per plant and per treatment were investigated. Drought depressed both nodulation and nitrogenase activity, and increassed the levels of growth inhibitors in roots. However ABA increased symbiotic $N_2$ fixation activity in control plants growing in field capacity, and also partially reversed the deletereous effects of drought. Gibberellin $A_3$ had no action on control plants but annulled the effects of ABA. There was no correlation between the levels of growth inhibitors promoted by exogenous application of growth regulators and the nitrogenase activity and weight of nodules.
Thus the diminution in nodulation and the reductive capacity of the system is not due to inhibitors accumulated in roots by effect of drought.

## RESUMEN

Plantas de soja (*Glycine max* L. MERRILL) que habían alcanzado máxima nodulación fueron sometidas a stress hídrico mediante el uso de polietilenglicol (PEG 6000) y asperjadas con Giberelina $A_3$ y/o ácido abscísico con el objeto de mitigar o simular el stress. Luego que las plantas fueron regadas nuevamente, se determinó el nivel de inhibidores del crecimiento endógeno en el sistema radical, la actividad nitrogenasa (tanto por raíz como por nódulo), el número de nódulos por planta y los pesos fresco y seco de nódulos y raíces por planta y por tratamiento. La sequía disminuyó la nodulación y la actividad nitrogenasa, aumentando los niveles de inhibidores endógenos en raíces. Sin embargo, el ABA aumentó la fijación simbiótica de nitrógeno en las plantas control crecidas en capacidad de campo, y también revirtió parcialmente el efecto deletéreo de la sequía. Giberelina $A_3$ no tuvo ningún efecto sobre las plantas control pero anuló la acción del ABA. No hubo correlación entre los niveles de inhibidores promovidos por aplicación exógena de reguladores del crecimiento, la actividad nitrogenasa y el peso de los nódulos.
En consecuencia, la disminución de la nodulación y la capacidad reductiva del sistema no es debida a los inhibidores acumulados en las raíces por efecto de la sequía.

*R.W. Racca, Instituto de Fitovirología, INTA, A.M. Bas 276, 5000 Córdoba, Argentina; J.A. Argüello y S.B. Nuñez, Fac. de Cs. Agropecuarias, U.N. de Córdoba; V. Luna y R. Bottini, Fac. de Cs. Exactas, Físico-Químicas y Naturales, U.N. de Río IV, 5800 Río IV; L. Frioni, Fac. de Agronomía, Universidad de la República, Montevideo, R.O. del Uruguay.*

## INTRODUCTION

It is known that in certain species water stress alters the endogenous levels of growth regulators (Kannangara *et al.*, 1982 and literature cited therein).

Abscisic acid (ABA) levels are frequently increased in the foliage of plants under water stress (Bottini and Luna de Stecco, 1982; Ismaili *et al.*, 1983; Walton, 1980), and its action is primarily related to water economy through its participation in the mechanism of stomatal opening and closing (Walton, 1980).

Milborrow and Robinson (1973) found a considerable increase in the ABA content of plants that had overcome a water stress period, and they suggested that the inhibitor could migrate from the foliage towards the roots.

However, in a previos work (Bottini *et al.*, 1981) we measured the levels of growth inhibitors in aerial parts and roots of soybean plants grown under drought conditions and treated with ABA and gibberellic acid ($GA_3$). Those results did not show any increase, either in the growth inhibitor pool or in the ABA content of roots of plants grown under drought, although the experiment was performed in the greenhouse and the evaluation of growth inhibitors made 10 days after the drought condition had been overcome by rewatering the plants.

In Leguminosae the lack of water also modifies nodulation and nitrogen fixation (Albrecht *et al.* 1984; Aparicio-Tejo and Sánchez-Díaz, 1982; Ismaili *et al.*, 1983), including the enzymatic reduction of leghemoglobin (Swaraj *et al.*, 1986).

Cytokinins as well as auxins are supposed to play an important role in the early stages of nodule formation and both gibberellins and ABA influence the phenomenon (Bottini *et al.*, 1981; Peuppke, 1986; Racca *et al.*, 1980; Williams and Sicardi de Mallorca, 1982).

High levels of gibberellin like substances have been detected in nodules of *Phaseolus lunatus* (Evensen and Blevius, 1981) and soybean (Williams and Sicardi de Mallorca, 1982), and several authors considered the possible control of nodulation by these phytohormones (Bottini *et al.*, 1981; Peuppke, 1986; Racca *et al.*, 1980; Williams and Sicardi de Mallorca, 1982). Nevertheless there is scarce information about the relation between the modification in endogenous growth regulators levels and the alterations of nodulation, both induced by drought.

If phytohormones regulate nodulation in some way, they could also control nitrogen fixation thus playing an important role, not only in water economy but in the nitrogen autotrophy of nodulated leguminosae.

The scope of the present work was to study the effect that drought periods would have on the levels of growth inhibitors in roots, and the nodultation and nitrogen fixation activities in soybean. Besides, we intended to reverse drought effects by exogenous application of ABA and GA3, which are supposed to be involved in the control of the phenomenon.

## MATERIAL AND METHODS

According to the technique described by Racca (1980), soybean seeds were aseptically sown in glass tubes and maintained in darkness at 32+- 2°C. Seventy two hs. after germination the seedlings were transferred to plastic sacs containing 20 ml of Street solution without N, and inoculated with *Bradyrhizobium japonicum* (strain 5019 MIRCEM 29W, Brazil), 3 x $10^7$ bacteria x $ml^{-1}$. Plants were grown in culture chambers at 30 ± 2°C, light intensity of 3600 lux (Sylvania Gro-lux) at the canopy level, 70-93% RH, and a photoperiod of 14 h of light and 10 h of darkness. Fifteen days after germination all plants showed completely formed nodules and were divided in two groups:

A. Plants that continued growing at field capacity;
b. Plants submitted to cycles of drought.

The physiological drought was obtained by the adition of polyethyleneglycol (PEG 6000) into the culture solution at an appropiate concentration able to produce a water potential of -9 bars. Simultaneously with the initiation of water stress, all plants (including those grown at field capacity), were sprayed with aqueous solutions of growth regulators, $GA_3$ (90% purity) and ABA (isomer ± cis-trans, 95% pure), Sigma Chemical Company, with 0.04% Tween 20, as follows:

**Plants grown at field capacity**
1. Distilled water (control)
3. $GA_3$ (75 ppm)
5. ABA (10 ppm)
7. $GA_3$ (75 ppm) + ABA (10 ppm)

**Plants grown under drought**
2. Distilled water (control)
4. $GA_3$ (75 ppm)
6. ABA (10 ppm)
8. $GA_3$ (75 ppm) + ABA (10 ppm)

Each treatment had 20 plants.

When plants of treatment 2 began to show yellow foliage (4 days of drought conditions), a second application was done at the same dosage. Forty eight h after the second treatment, the solution containing PEG 6000 was withdrawn and replaced with nutrient solution alone. When all plants showed symptoms of recovery (72 h after the osmoticum was eliminated), they were harvested to determine the endogenous levels of growth inhibitors and to measure the nitrogenase activity in roots and nodules. Also, the number of nodules per plant and per treatment and the fresh and dry weight of nodules and roots per plant and per treatment were registered.

The nitrogenase activity was determined with the technique of acetylene reduction to ethylene proposed by Hardy *et al.* (1973). Pieces of roots supporting nodules, and nodules alone, were incubated overnight in sealed 100 ml glass flasks in a $N_2$ atmosphere. Then, 10% of the $N_2$ was replaced with acetylene and allowed to incubate 1 more h at 32°C; aliquots of the atmosphere were taken and injected into a gas chromatograph with a Porapak column and a flame ionization detector, using $N_2$ as the carrier gas. Recorded peaks were compared with those obtained with pure ethylene, and the results expressed as umol of $C_2H_4$ x $ng^{-1}$ of dry weight of root (or nodule ) x $h^{-1}$.

To evaluate growth inhibitors 1 g dry weight of roots was homogenized and extracted with aqueous methanol 80% overnight at 4°C. After solvent evaporation under low pressure and temperature, the aqueous extract was purified by sequenced partition in $HNaCO_3$ (0.1 M, pH 8.0) and fresh diethyl ether (5 times per vol.), and then in $HNaCO_3$ 0.1 M pH 2.7 and fresh diethyl ether. The acidic ethereal fraction was dried over $Na_2SO_4$ and chromatographied into Silica Gel plates, 20 x 20 cm, 0.25 mm (Polygram Sil G $UV_{254}$, Macherey Nagel Company) with isopropanol: $OHNH_4$: $H_2O$ (100: 14: 6; v/v). Chromatographic sections were observed under UV light, eluted with pure methanol, and tested by the wheat coleoptile straight length growth bioassay (Bottini *et al.*, 1976). Ten 5 mm subapical coleoptile sections were floated onto 2 ml of a glucose-citric acid HNa $PO_4$-Ca $(NO_3)_2$ pH 5.2 buffer solution during 20 h at 25°C in darkness (under safety light) and with shaking.

Results were expressed as per cent of inhibition against a control. Control test with ABA(isomer ± cis-trans, 95% pure, Sigma Chemical Company), was performed under the same chromatographic conditions to determine its Rf value.

## RESULTS

### I. Effect of the different treatments upon the growth inhibitor pattern in roots of soybean plants.

Results concerning endogenous growth inhibitors are shown in figure 1 (A to H).

Roots of plants submitted to drought showed a strong accumulation of inhibitors (different from ABA), figure 1B, while, these regulators were absent in those grown at field capacity (Figure 1A).

Abscisic acid applied to plants grown at field capacity mimicked the effect of drought (Figure 1E); however the inhibitory activity found in roots was less than that observed under drought (Figure 1B). The same ocurred when ABA was applied to plants that underwent drought (Figure 1F), except for a peak of inhibition in the TLC zone in which ABA eluted (as a consequence of transport from the foliage?). Both ABA treatments (Figures 1E and 1F) gave a similar pattern of growth inhibitors (Rfs 0.7-1.0 on TLC), independently from the water conditions in which the plant had grown (the regulator masked the environmental effect).

Also both $GA_3$ treatments (Figures 1C and 1D) produced similar endogenous growth inhibitor patterns and not so different from those of ABA treatments. Applications of $GA_3$ in plants growing at field capacity produced accumulation of inhibitors of Rfs 0.6-0.8. Under drought conditions, $GA_3$ eliminated low Rf inhibitors, but this effect was much less over the high Rf inhibitor pool.

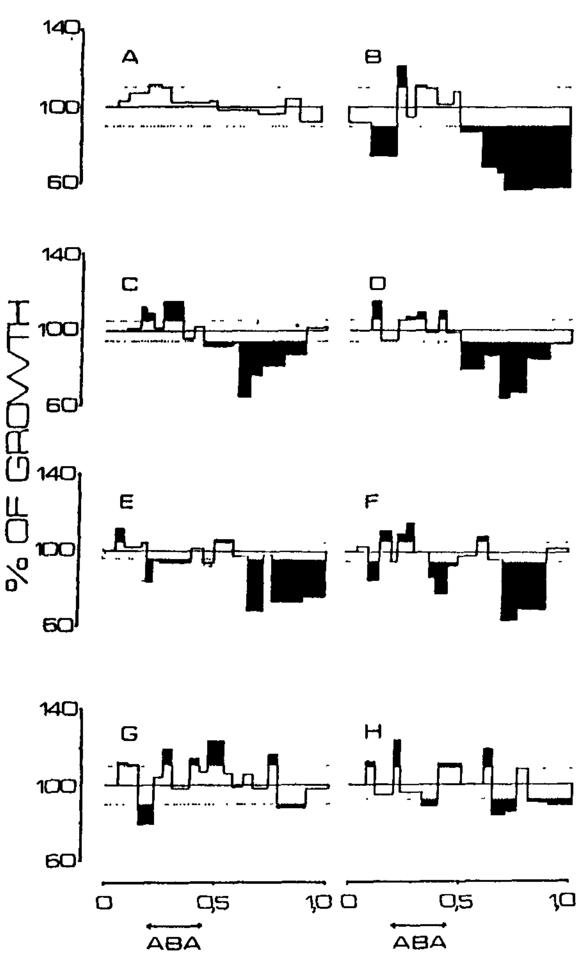

Figure 1. Levels of growth inhibitor activity, measured by the wheat coleoptile straight length growth bioassay, in root + nodules extracts of soybean plants. Ordinate: growth as per cent of controls (shadow zones represents significance levels, P 0.05). Abscissa: Rfs in TLC (Silica Gel G $UV_{254}$, 0.25 mm thickness, Macherey Nagel Co.) with isopropanol: ammonia: water (100: 14: 6). A, C, E, and G: plants grown at field capacity. B, D, F, and H: plants submitted to physiological drought with PEG 6000. A and B, plants sprayed with distilled water. C and D, plants sprayed with $GA_3$ 75 ppm. E and F, plants sprayed with ABA 10 ppm. G and H plants sprayed with $GA_3$ 75 ppm + ABA 10 ppm.

Surprisingly both regulators, ABA and $GA_3$, resembled the effect of drought increasing the growth inhibitor accumulation in roots of plants grown at field capacity. However they had the opposite effect on plants grown under drought conditions, diminishing (although moderately) their growth inhibitor levels.

The simultaneous sprays of ABA and $GA_3$ had almost no effect on the growth inhibitor patterns of roots of plants grown at field capacity (Figure 1G); a small inhibitory peak can be seen between Rfs 0.15-

0.22 that was not observed in the corresponding control and that was probably due to exogenous ABA or similar compounds. Nevertheless under the drought conditions, the normal growth inhibitor patterns were deeply diminished (Figure 1H) making the situation quite similar to that observed in control plants grown at field capacity (Figure 1A). Both ABA and $GA_3$, when applied separately, increased the levels of inhibitors of treated plants grown at field capacity. Under drought conditions, individual treatments with ABA or $GA_3$ moderately reduced the levels of growth inhibitors in relation to the controls, but applied together they exerted an additive effect making the situation similar to that of the control plants grown at field capacity.

### II. Effect of the different treatments on nodulation and nitrogenase activity.

As can be seen in Table I drought drastically affected the fresh weight of nodules and nitrogenase activity, expressed as per g dry weight basis either on nodules or roots plus nodules. The drought effect was less evident in relation to the number of nodules per plant probably because the drought cycle was performed after full nodulation.

Abscisic acid significantly increased the nitrogenase activity when plants were grown at eitber field capacity or under drought conditions, at field capacity the number and fresh weight of nodules per plant were also increased.

Gibberellin $A_3$ did not modified significantly any parameter measured in plants maintained at field capacity but it accentuated the deletereous effect in plants under drought.

At field capacity, when ABA and $GA_3$ were applied simultaneously, the results were the same as those obtained with $GA_3$ alone (antagonistic effect).

| Treatment | | Nodules per plant | | Nitrogenase activity (*) (µmol $C_2H_4$ mg d.wt$^{-1}$ h$^{-1}$) | |
|---|---|---|---|---|---|
| | | Average number | Average f. wt. (mg) | Nodules | Nodules + roots |
| Field Capacity | Water | 42 | 205 | 48.2 d | 4.54 d |
| | ABA | 56 | 312 | 134.5 e | 11.77 e |
| | $GA_3$ | 41 | 201 | 55.2 d | 4 51 d |
| | ABA + $GA_3$ | 55 | 180 | 46.7 d | 3.80 d |
| Drought | Water | 36 | 103 | 2.7 b | 0.16 b |
| | ABA | 33 | 94 | 24.4 c | 2.24 c |
| | $GA_3$ | 26 | 53 | 0.0 a | 0.0 a |
| | ABA $GA_3$ | 45 | 134 | 0.0 a | 0.0 a |

(*) Same letters within each column means no significant levels difference ($P \leq 0.05$) acording Tukey's stest.

Table I. Average number and fresh weight (f. wt ) of nodule per plant, and nitrogenase activity of soybean plants grown hydroponically under field capacity or drought and with different treatments with ABA and/or $GA_3$.

However, under drought, the simultaneous application of both regulators caused an increase in the number as well as in the fresh weight of the nodules with respect to the $GA_3$ treatment, but had no effect on the nitrogenase activity which continued being null.

## DISCUSSION

The results indicated that drought depressed both nodulation and nitrogenase activity, and also increased the levels of growth inhibitors in roots.

However, the hypothesis that the environmental stress could affect nodulation and nitrogenase activity through the endogenous growth inhibitor pool (depressing the overall plant growth leading to water economy) can not be true, because no correlation could be proved between nodule efectiveness and the levels of growth inhibitors. Moreover, when an inhibitor like activity similar to that found in roots of plants under water stress was promoted as a result of exogenous treatment with ABA and $GA_3$ to plants growing in conditions of good water supply, these parameters of symbiotic $N_2$ fixation did not decrease. Furthermore in the case of ABA application there was a significant increase in nodule number and nitrogenase activity, which in turn corroborates previous results (Racca *et al.*, 1980).

When plants under drought conditions were treated with ABA or $GA_3$, the growth inhibitors in the roots decreased, but this fact had no influence on nodulation. When both regulators were applied together the inhibitor levels became similar to the control at field capacity.

But this did not occur with nodulation and the reductive capacity of nodules themselves, which remain at level zero.

Moreover, applications of ABA under field capacity conditions resemble the effect of drought in relation to the pool of inhibitors found in roots, but those treatments had an opposite effect on nodulation and reductive capacity, which not only did not decrease but increased.

These results indicated that the effects of water stress on symbiotic $N_2$ fixation are not due to an increase in the levels of growth inhibitors. This assumption is reaffirmed if we consider that $GA_3$, which is antagonistic of ABA in many physiological phenomena, had little or no effect on plants grown at field capacity and diminished nodulation and reductive capacity in plants that underwent drought. This could be explained as an indirect effect.

Abscisic acid would depress growth of aerial parts leading the assimilate flux towards roots and improving nodulation and nitrogenase activity. On the contrary, $GA_3$ applications would lead photoassimilates to remain in aerial parts depressing nodulation and nitrogenase activity under environmental conditions of stress.

However this overall explanation may not be so simple, if we keep in mind that $GA_3$ affected the nitrate reductase activity in nodules. Also in *Medicago sativa*, *in vitro* treatments with pesticides that decrease nodulation depressed the levels of gibberellin-like activity in nodules (Correa *et al.*, 1987, personal communication to R.B.).

Under the conditions used, during the drought period further nodules might have developed. The fact that there were fewer nodules in the stressed plants than in the controls suggests either that nodules were lost during the treatment period or that further nodulation was suppressed. The latter is more likely, since PEG is known to affect infection of root hairs by rhizobia (Zahran and Sprent, 1986). As some of the references quoted by these authors show, young and mature nodules may have completed hormone contents. Therefore, since the two treatments are likely to have affected the age distributions of the nodule populations, differences in hormone content were likely for this reason, besides from the effects of the treatments given. However, this statement does not invalidate the possibility that this situation could have really happened under field conditions, and the most important fact observed was that ABA reversed drought effects independently of its nature.

## BIBLIOGRAPHY

Albrecht, S., Bennet, M., Boote, K., 1984. Relationship of nitrogenase activity to plant water stress in field-grown soybeans. Field Crop Res. 8, 61-71.

Aparicio-Tejo, P., Sanchez-Díaz, M., 1982. Nodule and leaf nitrate reductase, and nitrogen fixation in *Medicago sativa* L. under water stress. Plant Physiol. *69*, 479-482.

Bottini, R. Luna de Stecco, M. V., 1982. Allelopathy and endogenous inhibitors in sunflower (*Helianthus annus* L.): effect of drought. Phyton *42*, 67-72.

Bottini, R., de Bottini, G. Correa, N., 1976. Changes in the levels of growth inhibitors and gibberellin-like substances during dormancy of peach flower buds. Phyton 34, 157-167.

Bottini, R., Racca, R. W., Argüello, J. A., Collino, D., Tizio, R., 1981. Levels of growth inhibitors in soybean (*Glycine max* L. Merrill) cv. Lee, in response to drought, gibberellic acid and abscisic acid. Phyton *41*, 97-102.

Evensen, K., Blevius, D., 1981. Differences in endogenous levels of gibberellin-like substances in nodules of *Phaseolus lunatus* L. plants inoculated with two Rhizobium strains. Plant Physiol *68*, 195-198.

Hardy, R., Burns, R., Holsten, R., 1973. Application of the acethylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. *5*. 47-81.

Ismaili, M., Briske, B., Weaver, W., 1983. Nitrogen fixing activity of water stressed Siratro. Agron. J. *75*, 649-653.

Kannangara, T., Durley, R. C., Simpson, G. M., Stout D. G., 1982. Drought resistance of *Sorghum bicolor*. 4. Hormonal changes in relation to drought stress in field grown plants. Can. J. Plant Sci. *62*, 317-330.

Milborrow, B. V., Robinson, R., 1973. Factors affecting the biosynthesis of abscisic acid. J. Exptl. Bot. *24*, 537-548.

Peuppke, S. B., 1986. Physiology of nodule initiation in the nitrogen-fixing legume-rhizobium symbiosis. Physiol. Plant. *67*, 262-266

Racca, R. W., Bottini, R., Argüello, J. A., Chessa, A., Collino, D. J., Tizio, R., 1980. Influencia de un período de stress hídrico y algunos reguladores del crecimiento sobre la nodulación de dos cultivares de soja. Rev. Fac. Agr. UNBA *1*, 25-32.

Swaraj, K., Topunov, A., Golubera, I., Kretovich, V., 1986. Effect of water stress on enzymatic reduction of leghemoglobin in soybean nodules. Soviet Plant Physiol. *33*, 70-74.

Walton, D. C., 1980. Biochemistry and physiology of abscisic acid. Annu. Rev. Plant Physiol. *31*, 453-489.

Williams, P. M., Sicardi de Mallorca, M., 1982. Abscisic acid and gibberellin-like substances in root nodule of *Glycine max*. Plant and Soil 65, 19-26.

Zahran, A., Sprent, J., 1986. Effects of sodium chloride and polyethylenglicol on root-hair infection and nodulation of *Vicia faba* L. plant by Rizobium-leguminosarum. Planta *167*, 303-309.