Morphogenesis in sunflower (*Helianthus annuus* L.) as affected by exogenous application of plant growth regulators

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SUMMARY

The effect of the plant growth regulators (PGRs) N^6-benzyladenine (BA), α-Naphthaleneacetic acid (NAA) and Gibberellic acid (GA_3) on morphogenesis of sunflower (*Helianthus annuus* L) was studied PGRs were applied at the beginning of capitulum development, where floret primordia were still not determined, at dosis of 45 µg of NAA day^{-1} during 10 days, or 45 µg of BA day^{-1} during 10 days, or 45 µg of GA_3 day^{-1} during 5 days

Compared with untreated plants, NAA was not able to produce any significant change. GA_3 was effective in increasing the length of stem internodes and accelerating the onset of floral development by 25.0 %. BA showed the most significant effect on plant development, increasing the leaf area per plant by 38.0 %, the stem dry weight by 93.0 % and changing capitulum morphology and hence disc floret development. BA-treated plants showed an increase in the number of floret primordia of 17.0 % associated with a significant promotion of the receptacle expansion before the begining of the floret differentiation period

Key words: *Helianthus annuus*, morphogenesis, growth regulators

Hernández, L.F., 1996. Morfogénesis en girasol (*Helianthus annuus* L) en respuesta a la aplicación exógena de reguladores de crecimiento. Agriscientia XIII : 3-11.

RESUMEN

Se estudió el efecto de la aplicación exógena de los reguladores de crecimiento vegetal (RCV) N^6-Bencíladenina (BA), Acido α-Naftalenacético (NAA) y Acido giberélico (GA_3) sobre la morfogénesis en el girasol (*Helianthus annuus* L) Los RCV fueron aplicados durante el desarrollo incipiente del capítulo, es decir cuando los primordios florales aún no habían terminado de diferenciarse. Cada planta tratada recibió 45 µg de NAA $día^{-1}$ durante 10 días o 45 µg de BA $día^{-1}$ durante 10 días o 45 µg de GA3 $día^{-1}$ durante 5 días NAA no produjo cambios significativos tanto en el desarrollo del tallo como en la morfología del capítulo GA_3 produjo un aumento en la longitud de los entrenudos y aceleró el desarrollo floral en un 25,0%. BA fue el que mostró el mayor efecto sobre el desarrollo de las plantas, aumentando la superficie foliar en un 38,0%, el peso seco del tallo en un 93,0% y produciendo cambios en la morfología el capítulo y en el desarrollo floral Las plantas tratadas con BA presentaron un 17,0% de aumento en el nú-

Fecha de recepción: 1/3/96; fecha de aceptación: 18/12/96.

mero de primordios florales, el cual se pudo asociar con la significativa expansión de la superficie del receptáculo en una etapa previa al comienzo de la diferenciación floral.

Palabras clave: *Helianthus annuus*, morfogénesis, reguladores de crecimiento.

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INTRODUCTION

Exogenous application of plant growth regulators (PGRs) produces different responses in reproductive plant development, ranging from acceleration of floral development by gibberellins (Pharis and King, 1985) to the complete inhibition of flowering by morphactins (Schneider, 1970). So this method has been widely used to gain understanding of hormone action on flowering and leaf and flower morphogenesis (Bernier *et al.*, 1981; Bradford and Trewavas, 1994; Goodwin *et al.*, 1978; Kinet *et al.*, 1985; Reid, 1993).

Cytokinins have been positively associated with flower and bud formation in many plants (King *et al.*, 1990; Leonard and Kinet, 1982). The exogenous application of BA has proved to be effective in increasing the size of the shoot apical meristem and the size and number of floral organs in different species (Crosby *et al.*, 1981; Jeffcoat, 1977; Mauseth, 1976; Napier *et al.*, 1986). Benzyladenine (BA) promoted and in some cases accelerated inflorescence differentiation in sunflower (Palmer and Hernández, 1988). Cytokinins can induce cell division and has been suggested that the morphogenetic effects regulated by cytokinins are related to enhanced cell division activity (Francis and Lyndon, 1985). It has also been observed an increase in meristematic activity in the peripheral zone in shoot apical meristems of *Chenopodium* and an increase in the cell size after the application of kinetin (Seidlová and Krekule, 1977).

Cytokinins are involved in the endogenous mechanisms of mobilization of nutrients to developing leaves and growing fruits (Horgan, 1984). They can cause mobilization of metabolites, mainly amino acids and photoassimilates, to the treated site (Havelange *et al.*, 1986; Leonard *et al.*, 1983). The importance of BA for floral morphogenesis in tissue culture of sunflower explants has also been demonstrated (Paterson, 1984; Power, 1987).

Applied auxins can promote or inhibit flower initiation although in many reported cases of promotion of flowering, the effect was confined to photo-inductive conditions that were close to the threshold for flowering (Bernier, *et al.*, 1981, Cline, 1994; King *et al*, 1990). Modification of floral organs arrangement and phyllotaxy by auxin application has been found in different species (Bernier *et al.*, 1981; King *et al*, 1990, Riov and Bangerth, 1992; Yang *et al*, 1993)

Gibberellins (GAs) generally increase stem elongation and leaf area and decrease root growth (Bernier *et al.*, 1981). Specifically GA_3 increases the rate of cell division in the central zone of the shoot apex and stimulates mitotic activity in sub-apical tissues accelerating the flower development (Bernier *et al*, 1981; Bradford and Trewavas, 1994, Pharis and King, 1985).

Exogenous application of PGRs in crop plants and the knowledge of how the shoot apical meristem responds to differents compounds and concentrations can be used as a tool for physiological studies oriented to crop improvement. This paper reports the results obtained in experiments were the effect of three PGRs externally applied during early stages of floral development in sunflower were investigated

MATERIALS AND METHODS

Plant cultivation

Sunflower plants, cultivar Dekalb G100 were grown in 2.0 L plastic pots using 1 1:1 peat:perlite:loam potting soil in a controlled environment cabinet at long day (LD) photoperiod (18 + 6 Light/Darkness) and 28 ± 2 °C. Plants were watered daily and fertilized once a week with 20-20-20 fertilizer. Minor mineral elements were also added two times a week. During the photoperiod the quantum flux (400-700 nm) was provided by Sylvania cool white (105 W HD) fluorescent tubes and 40 W incandescent lamps, with an intensity at the canopy level of 320 µmol $m^{-1}s^{-2}$

Application of PGRs

N^6-benzyladenine (BA) α-Naphthaleneacetic acid (NAA) and Gibberellic acid (GA_3, 90% minimum content, Sigma Chemical Co., St. Louis, USA) solutions were prepared in aqueous ethanol 5% (v/v). The concentrations of the different PGRs were selected following Hernández (1988). Previous experience (Hernández, 1988) proved the ethanolic solution to be inocuous for plant development. Experiments started at Floral Stage (FS) 2, about 21 days after emergence (DAE), when the apical dome broadens and flattens and the differentiation of involucral bract primordia is still undetected (Marc and Palmer, 1981). Each PGR solution was carefully injected into the unfolded leaves of the terminal bud of each plant using a microsyringe. The moment for the application was the same in all treatments, namely 11 AM in the light cycle. The amount of each PGR applied and the timing of application was scheduled as follows:

Treatment	PGR and amount applied
CONTROL	5% (v/v) ethanol. 150 µL plant^{-1}day^{-1} for 10 days.
NAA	a-Naphthaleneacetic acid. 45 µg plant^{-1} day^{-1} for 10 days.
BA	N^6-benzyladenine. 45 µg plant^{-1}day^{-1} for 10 days.
GA_3	Gibberellic acid. 45 µg plant^{-1}day^{-1} for 5 days.

Care was taken to avoid the solutions making contact with the soil by covering each pot with a sheet of plastic foil. Each apical bud received a total amount of either 450 µg of NAA, or 450 µg of BA, or 225 µg of GA_3 during the experimental period. Control plants were treated only with aqueous ethanol (5%, v/v) .

Measurements

Starting at the time of PGRs application and at intervals of 5-8 days, 5 plants per treatment were taken for assesment of the developmental stage of the capitulum, receptacle surface area, leaf area, stem length and root, stem, and leaf dry weight. The development of the apex was followed by periodic sampling, starting 3-4 DAE using the ten stages classification of Marc and Palmer (1981). Plant leaf area was calculated by weighing paper replicas of each leaf. Mean leaf and stem dry weight was obtained by drying the samples in an oven at 60°C for 48 h. For root dry weight, the plants were carefully removed from the pot and the soil washed away under running tap water. Then the roots were wiped between sheets of absorbent paper and placed in an oven at 60 °C for 24 h.

On the same batch of plants, the capitula were dissected out and the diameter of each receptacle measured in 3 planes of radial symmetry. The average value was obtained and the surface area of the receptacle calculated. Floret count on each capitulum was made using the replica method developed by Green and Linstead (1990). A mold of the capitulum surface was made, a resin replica obtained and the number of floret and the distance between the centers of symmetry of floret primordia in FS 5 measured. Some of this replicas were gold-coated, examined in a JEOL JSM-35CF scanning electron microscope (SEM) at 10 kV and photographed Twenty five plants were harvested 10 days after FS 10 (62 DAE) and the number of involucral bracts, incipient ray florets and row parastichies in the capitulum recorded. At 50 DAE, few plants for all treatments were placed in a glasshouse and left to grow until fruit maturity.

Statistics

The experiments were carried out using a randomized block design. The data for two replicate experiments were pooled and subjected to an analysis of variance using appropriate software. When F was significant ($P < 0.05$), the least significant difference (L.S.D.) for the comparison of means was determined for each sample (Steel and Torrie, 1980)

RESULTS

Quantitative changes observed in the vegetative and reproductive development of plants are shown in Fig. 1. The application of BA produced an increase in leaf area (Fig. 1a), leaf dry weight (Fig. 1b) and stem dry weight (Fig. 1c). Compared with the other treatments there was also a significant increase in receptacle surface area (Fig 1d) and a small increase in root dry weight (Fig 1e). The onset of flowering was not significantly affected, but the rate of floret differentiation changed with each treatment (Fig. 1f). Some leaves of the BA-treated plants showed an abnormal appearance resembling to some extent calcium deficiency symptoms (Blamey *et al.*, 1987) particularly by a crinkled appearance of the younger ones.

In some cases (19.1% of the total), BA induced the formation of "secondary inflorescence centers" that were distributed around the natural rim of the receptacle. Those abnormalities were apparently produced by the centripetal differentiation of floret initials towards the spatial centers of these areas (Fig. 2a). Compared with the controls (Fig. 2b) these capitula also sho-

wed a significant increase in the number of rows of floret primordia at the receptacle rim (Table 1) The classic Fibonacci arrangement of floret parastichies in these plants was not observed (Fig. 2a). In other plants (12% of the total) disc floret initials developed in the receptacle center without any apparent continuity with the peripheral florets. Similar results had been obtained previously (Hernández, 1988, 1990; Palmer and Hernández, 1988). The cytokinin treatment also increased the number of disc floret rows in 29% of the treated LD plants (Table 1). In these cases, the receptacle surface generally became undulated or wave-shaped and the regular floret arrangement was lost both at the center and rim of the receptacle (Fig. 2). BA-treated plants grown up to maturity showed an increased number of fruits of about 25%. In these cases more than 50% of the total fruits were empty at the moment of counting.

GA_3 treatment did not produce significant changes in inflorescence growth except that there was an increase in the rate of floral development of about 0.10 units of FS day^{-1} The rate increased from 0.25 units of FS day^{-1} in the control to 0.35 units of FS day-1 in the GA_3-treated plants (Fig. 1f). The final stage of inflorescence formation (FS 10) was then obtained 9 days earlier than in the control (Table 1 Fig. 1f) In NAA-treatted plants, the completion of inflorescence development was delayed in 3 days (Fig. 1f). The stem dry weight was also significantly increased in the GA_3 treatment (Fig. 1c) as a result of considerable stem enlargement (Fig. 1g) without any significant increase in leaf growth (Fig. 1a-b).

GA_3 application accelerated floret diferentiation (Fig. 1f) but did not produce changes in the number of floret rows (parastichies) in the capitulum (Table 1). Compared with the controls NAA-treated plants did not show any change in capitulum morphology Treatment with BA stimulated a significant increase in the receptacle surface area (Fig 1d) and in the total number of floret primordia without producing any significant variation in the interprimordial spacing or floret size (Table 1) These responses were observed in 82% of the BA-treated plants.

The application of NAA did not produce any significant response in the development of vegetative structures (Fig. 1a-e, Fig. 1g). Small differences observed in the data were not proved to be statistically significant. NAA-treated plants were morphologically similar to the controls For these reasons the application of NAA was not investigated any further. The number of involucral bracts and ray florets developed under different treatments, did not show any significant difference (Table 1).

DISCUSSION

In this work it is shown that the BA treatment was effective in changing the pattern of disc florets and also altering the floret row number in the capitulum. BA increased the number of parastichies and hence the total number of florets developed in the capitulum Also, the natural Fibonacci arrangement of the floret rows was disrupted

In the sunflower capitulum the initiation of floret primordia is a result of a change in the rate and plane of cell division at the surface and sub-surface level (Hernández, 1988, 1990, Hernández and Green, 1993). Each primordium arises as a consequence of a change in orientation of cell division, from normal to the receptacle surface (anticlinal) to parallel to the

Table 1. Effect of the external application of NAA, BA and GA_3 on the development of the inflorescence of the cv. Dekalb G100 growing under a LD photoperiod.

Treatment	Involucral bracts (Nº)	Ray florets (Nº)	Row pair	Frequency of row pair (%)	DAE to attain FS 10	Disc florets per long row (Nº)	Disc floret width (µm)	Spacing between florets (µm)
CONTROL	81 ± 3 a	57 ± 3 a	55/89	100	47 1 ± 1.2 a	34 1 ± 3 3 a	113 0 a	112 5 a
NAA	79 ± 5 a	59 ± 5 a	55/89	100	50 3 ± 1.1 a	32 4 ± 2 7 a	111 1 a	115 5 a
BA	83 ± 4 a	65 ± 7 b	55/89	71	42.2 ± 1 3 b	39 9 ± 9 5 b	108 5 a	114 1 a
GA_3	80 ± 2 a	53 ± 4 a	55/89	88	38 1 ± 1 4 c	28 5 ± 2 9 a	114 5 a	125 6 a

BA: N^6-benzyladenine, NAA: α-Naphthaleneacetic acid; GA_3: Gibberellic acid.
The percentages of the row pairs (Frequency %) are calculated from 10 plants used in each experiment on 4 replicates The complement (100 % - Frequency %) is the percentage of abnormal plants, plants with 1 step more or less in the Fibonacci sequence or plants or with increased number of rim florets. Values followed by the same letter in the same column do not differ significantly ($P < 0.05$) by the Duncan's multiple range test

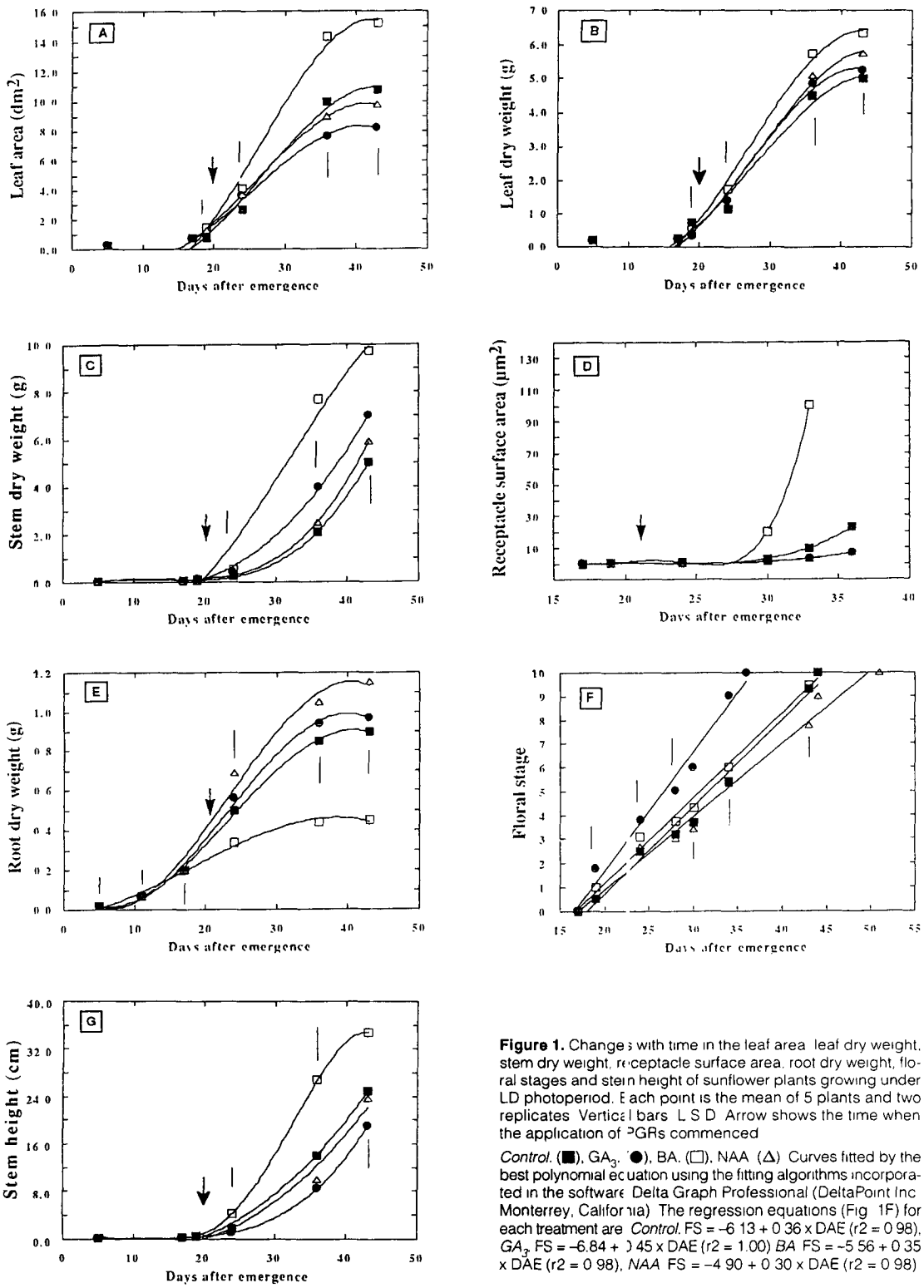

Figure 1. Changes with time in the leaf area, leaf dry weight, stem dry weight, receptacle surface area, root dry weight, floral stages and stem height of sunflower plants growing under LD photoperiod. Each point is the mean of 5 plants and two replicates. Vertical bars, L.S.D. Arrow shows the time when the application of PGRs commenced.

Control. (■), GA_3. (●), BA. (□), NAA (Δ). Curves fitted by the best polynomial equation using the fitting algorithms incorporated in the software Delta Graph Professional (DeltaPoint Inc. Monterrey, California). The regression equations (Fig. 1F) for each treatment are: *Control.* FS = −6.13 + 0.36 x DAE (r^2 = 0.98), GA_3 FS = −6.84 + 0.45 x DAE (r^2 = 1.00) *BA* FS = −5.56 + 0.35 x DAE (r^2 = 0.98), *NAA* FS = −4.90 + 0.30 x DAE (r^2 = 0.98).

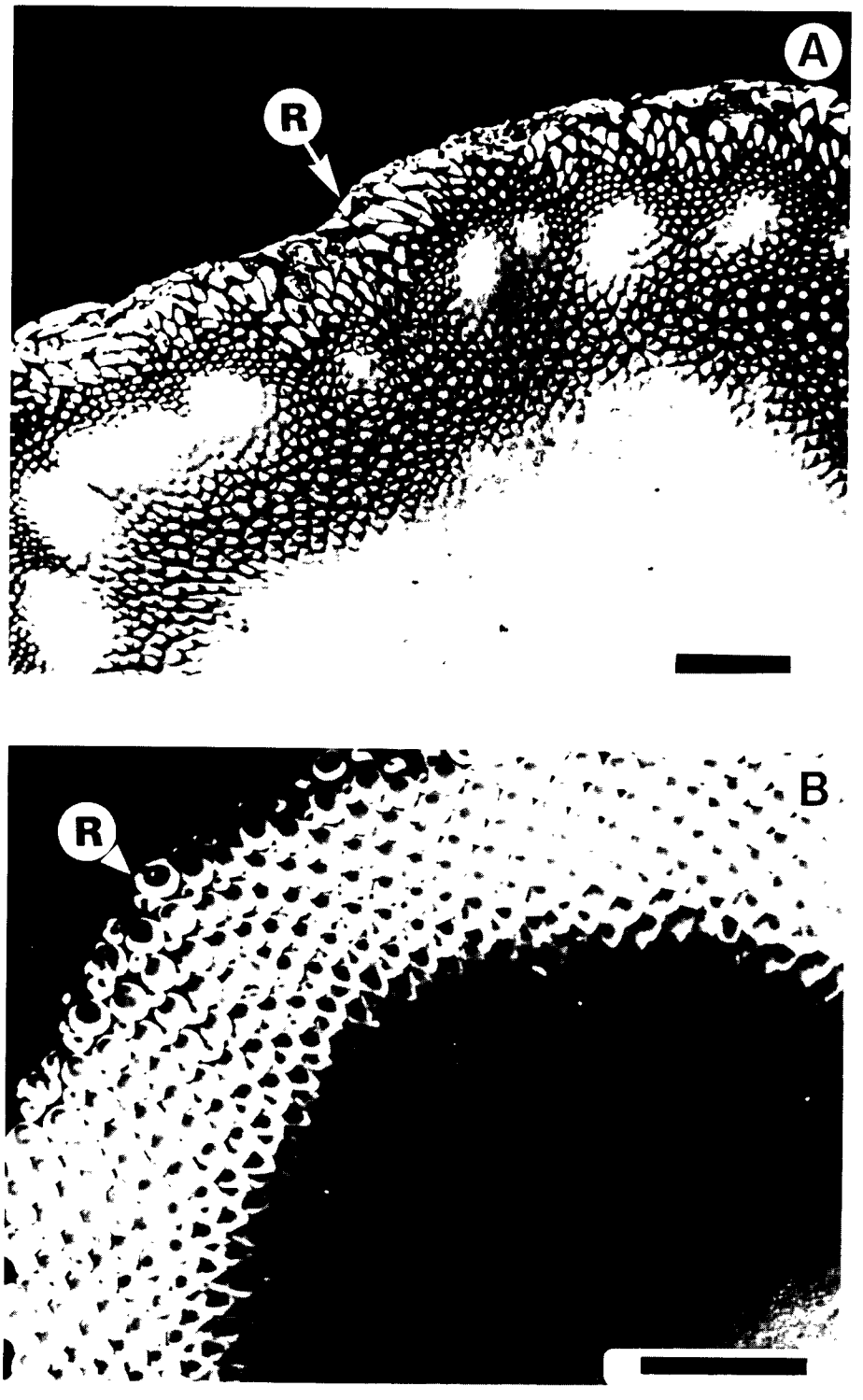

Figure 2. ***A.*** Scanning electron micrograph of the receptacle of a BA-treated plant, 34 DAE, showing the irregular arrangement of floret initials and the formation of raised and convoluted areas at the capitulum rim (R) The number of left-handed rows at the rim is 217 This floret pattern does not conform with the Fibonacci series of numbers ***B.*** Capitulum of a control plant of the same age as in ***A.*** Bar = 500 µm

surface (periclinal) (Hernández, 1988; Palmer and Hernández, 1988).

The application of cytokinin is known to produce an increase in the mitotic activity of vegetative meristems (Chailakhyan, 1982; Kinet *et al.* 1985; King *et al.*, 1990) and an increase in the concentration of cellular proteins, RNA and DNA (Naito *et al.*, 1978). The results obtained here suggest that BA could have two parallel effects: first it could promote an increase in the cell division activity in all the receptacle tissue leading to its expansion (Fig. 1d) and second, an increase in periclinal cell divisions at the sub-surface level, resulting in an enhancement of the potential sites for the production of more florets and then more primordia being produced per unit of receptacle surface area. It is then possible that the increase in size of the receptacle accompanied by a significant increase in floret primordia number in the BA-treated plants (Table 1) could also be induced by an accelerated mobilization of assimilates towards the capitulum. It has been shown that in *Pharbitis*, the promotion of flowering by cytokinin was a result of a redistribution of assimilates towards the shoot apex (Ogawa and King, 1979). Also in sunflower the removal of involucral bract primordia at early stages of capitulum development produced a significant increase in capitulum size and floret primordia number (Hernández, 1993).

For technical reasons the arrangement of the involucral bracts was not studied. Table 1 shows that no significant differences were found in the final number of involucral bracts between treatments. Therefore whether the changes observed in the disc floret pattern could also have been produced in the initial arrangement of the involucral bract primordia is not known.

The different percentage of plants that responded to the application of BA (71%) and GA_3 (88%) compared with the control and the NAA treatment (100%, Table 1) could be the result of the stage of development of the capitulum at the time when the PGRs were applied. The time of application was selected by sampling and it is difficult to ensure that all the plants treated were in the same stage of floral development. However the uniformity of the experimental material and procedures used ensured that approximately 80-85% of each batch of plants were only ± 1-2 days displaced from the stage of flowering calculated, using the linear regression of floral development (Marc and Palmer, 1981). The efficacy of BA could depend on the physiological status of cells receiving the chemical. If the receptacle cells were in an actively dividing phase (i.e. after floral initiation), BA could stimulate cell division and hence may induce an increase in the number of florets, but if the cells were not rapidly dividing or in a G1 phase, BA could stimulate expansion (Bernier *et al.*, 1981; Francis and Lyndon, 1985).

Considering the results shown here, three hypotheses are presented to explain the effect of BA on receptacle morphogenesis. First, it is clear that an enlargement of the receptacle size is produced when BA is applied before the receptacle meristem has been commited to differentiate floret primordia (FS 5). BA can enhance cell division activity leading to the production of a large number of cells in the receptacle tissue. Because of this, there would be an increased mobilization of assimilates to the capitulum, produced by an enhancement of the sink strength of the capitulum tissue (Patrick, 1976). Even though it is not known if an increase in the total cell number in the capitulum can cause an increase in the number of floret primordia developing in the receptacle, this can demonstrate that the number of florets in the sunflower capitulum is not fixed but can be chemically manipulated

Second, if cytokinins are a controlling factor in the development of the capitulum, then the levels of endogenous cytokinins may be limiting the potential total number of floret primordia. Endogenous cytokinins may play a major role in the control of early stages of floral development in tomato (Leonard and Kinet, 1982, Napier *et al* , 1986). Although the concentration of endogenous cytokinin was not determined in this work, it is worth mentioning previous findings (Sitton *et al.*, 1967) where the cytokinin production by sunflower roots was measured in root exudates from the early stages of development It was found that the concentration of kinetin was higher during initiation of floret development (28.0 μgL^{-1}) in comparison with the vegetative stage (3.8 μgL^{-1}) or the anthesis period (3 5 μgL^{-1}).

Third, if the generation of each floret primordium originates from a predetermined number of cells laid down at an early stage of development, as has been demonstrated for maize (Coe and Neuffer, 1978; Steffensen, 1968), then the increase in the total number of cells induced by BA, could create new groups of cells committed early in the development of the capitulum to becoming floret primordia

CONCLUSION

In the present study it has been established that the exogenous application of two PGRs were able to induce important changes in capitulum growth and development during flowering. Gibberellic acid accelerated the rate of stem growth and floret develop-

ment without producing any change in the phyllotactic pattern of the floret primordia N^6-benzyladenine, induced changes of the disc floret primordia pattern in the receptacle and incresed the number of floret primordia.

BA applied at an early stage of capitulum development showed a high potential for further studies. The results obtained with BA show that an increased generation of disc florets can occur independently of the phyllotactic control mechanism that governs the position of leaf primordia. This implies that the generation of floret organs can be isolated from the controlling influence of the position of other shoot organs such as leaves and involucral bracts already present in the plant.

ACKNOWLEDGMENTS

This research was supported by the Secretaría General de Ciencia y Tecnología - UNS. L.F.H. is member of the Scientific Career of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC, La Plata, Argentina).

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