AGRISCIENTIA., VIII: 9-14.

Hormonal regulation of dormancy in garlic (*Allium sativum* L.) cv Rosado Paraguayo

Argüello J., Ledesma A. and Bottini R.

ABSTRACT

In order to establish the location of dormancy mechanism and the role played by different growth regulators upon it, garlic seed cloves (in dormant, end of dormancy; and sprouting periods) were cultured in vitro with a nutrient media/or several phytohormones. Also GA-like (free-acid and glucosyl-conjugate), auxin-like, and growth inhibitor-like substances were evaluated. Results showed that cytokinins were active during differentiation processes along dormancy (although the auxin role can not be discarded); GAs showed to be important at the end of dormancy perhaps throughought activation of carbohydrates mobilization. Auxin-like activity was higher significant by the time tissues expansion began (once the dormant condition has been overcome). Phytohormones (ABA included), should be important in the whole differentiation/ growth process due to the noticeable effect obtained with in vitro cultured explant when they were applied all mixed together. Thus, sprout lest would underpass several steps of differentiation (wich depend on hormonal, nutritional, and physico-chemical influences from the storage leaf) before sprouting. This dependence is more tight during dormancy, and become loose post-harvest time goes by.

Abbreviations: ABA, abscisic acid; BA, benzyladenine; n-BuOH, butanol; EtOAc, ethyl acetate; GAs gibberellines; IAA, indol 3 acetic acid; MeOH, methanol; PVPP, polyvinylpolypirrolidone.

Key words: dormancy - Giberellins - benzyladenine - garlic - abscisic acid

RESUMEN

Para establecer la localización del mecanismo de control de dormición y el papel de diferentes reguladores del crecimiento sobre el mismo, dientes semilla de ajo (dormidos, en salida de dormición y en brotación) se cultivaron *in vitro* con medio nutritivo y diversas fitohormonas. También se evaluó la presencia de sustancias tipo GAs (fracciones libres ácidas y conjugados glucosídicos), tipo auxinas y tipo inhibidores del crecimiento. Los resultados mostraron que las citocininas fueron efectivas durante dormición en los procesos de diferenciación (aunque no se puede descartar el papel de las auxinas); las GAs fueron importantes hacia salida de dormición, quizás mobilizando carbohidratos. La actividad tipo auxinas fue altamente significativa cuando comenzaba la expansión de tejidos (una vez que la condición durmiente había desaparecido) El conjunto de fitohormonas (ABA incluído) parecería ser importante en los procesos de crecimiento/diferenciación, debido al efecto notable de su aplicación en forma de mezcla sobre los explantos cultivados *in vitro*. Así, las hojas embrionarias deben superar diversos pasos de diferenciación (que dependen de influencias de naturaleza hormonal, nutricional y físico-química por parte de la hoja de reserva) antes de brotar. Esta dependencia es más estrecha durante la dormición y se hace progresivamente más débil a medida que transcurre el período de post-cosecha.

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Fecha de recepción: 11/6/91; fecha de aceptación: 30/9/91.

INTRODUCTION

There is some controversy about the physiology of dormancy in garlic seed cloves, related to where the mechanism a control is located. Kothari (1979) claimed that the regulation is done through the storage leaf, while Stritzke and Peters (1970) suggested that the sprout leaf itself controls its own growth rate.

Growth of isolated plant parts in liquid or agar media is useful to locate the mechanism of dormancy. Some authors have separated embryos their from seeds (Sánchez and De Miguel, 1983) or buds from the entire plant (Borkawska and Powel, 1979; Shibakusa, 1979), and submitted them to different *in vitro* culture conditions in order to clarify the problem.

Also addition of different growth regulators to the culture medium has been employed to study bud dormancy in fruit trees (Weinberger, 1969); (Altman and Goren, 1974; Naver and Boswell, 1981), potato tubers (Rappaport *et al.*, 1965) and onion bulbs (Thomas, 1969; Mahotiere *et al.*, 1976).

In a previous work (Argüello *et al.*, 1983) involvement of gibberellin (GA^{**})-like substances in the control of dormancy of garlic seed cloves was studied. However no discrimination was made in relation to a particular GA status of sprout storage leaves, and levels of conjugated forms were not evaluated Rakhimbaev and Ol'Shanskaya (1976) reported that higher levels of glucosyl-conjugated GAs ocurred during garlic seed cloves dormancy which in turn changes to free acid GA-like substances at the begining of active growth, as a probable result of interconversion. However, determinations were done with whole seed cloves.

In relation to auxins their role in bulbs and tubers is almost unknown (Thomas, 1981). although exogenous auxins prolonged dormancy in potato (Goodwing, 1963; Tizio, 1982).

Following the general theory for control of dormancy in tree buds proposed by Phillips (1962), Thomas (1981) suggested that the process in onion is controlled by a balance between growth prometers and inhibitors. In garlic, however, growth inhibitor substance levels did not characterize the dormancy status (Argüello *et al.*, 1983); a similar situation in found in onion bulbs (Stow, 1976).

Also, a possible hypothesis about a "physico-mechanical" role of storage leaf can not be rouled out, as proposed by Lewak (1979) for dormant seeds, which in turn excluded the hormonal control. Based in all the above evidences the aim of this work was a) to establish where are located the factors that control dormancy in garlic seed cloves; b) to determine the role played by some phytohormones in the posible physiological control of the process; and c) to prove if the storage leaf itself can constitute a "physico-mechanical" factor of dormancy.

MATERIAL AND METHODS

Garlic (*Allium sativum* L. cv Rosado Paraguayo) bulbs were harvested during november 1988 and kept at 20°C, 30-40% RH, and darkness. Three sets of seed cloves were selected along the storage period by uniform weight (2.0+/-0.2 g) and according to previous phenologic results (Argüello *et al.*, 1983) at: 1) dormancy, 10 days after harvesting; 2) end of dormancy, 60 days after harvesting, and 3) sprouting, 240 days after harvesting.

In vitro culture experiments

Seeds cloves were sterelized 15 min with Ca hypochloride 6,4% and then washed 5 times with distilled water. After that, sprout leaf was thoroughfully separates from storage leaf, and sequentially kept 20 min in sterilized distilled water plus Triton X-100, washed 5 times with water kept 10 sec in absolute ethanol, then sterilized for 20 min with Ca hypochloride 6,4%, and finally washed 5 times with water. Sprout leaves were placed in Petri dishes over Wathman paper or in culture tubes with semi-solid agar medium, and the following experiments were performed:

Experiment a. In this experiment 4 treatments were performed in wich both, dormant (set 1) and sprouting (set 3) sprout leaves were aseptically cultured in 2 different media: a semi-solid nutrient-free agar medium, and a "AZ" (Abbo El-Nil and Zettler, 1976) nutrient enriched semi-solid culture medium.

Experiment b. The various treatment included:

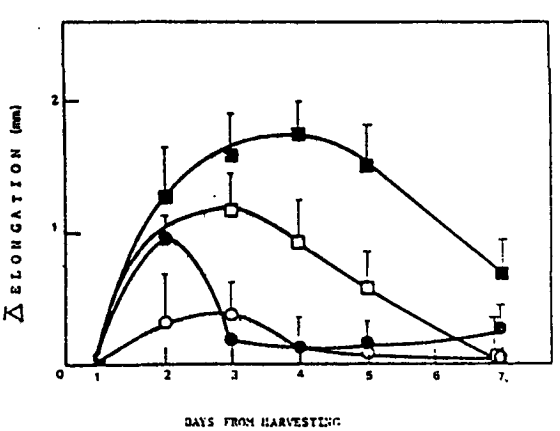

Figura 1: Growth rate of isolated sprout leaves during dormancy and post-dormancy. Dormant sprout leaves place on nutrient-free (-O-) and "AZ" nutrient-enriched (-O-) semi-solid agar media; non-dormant sprout leaves places on nutrient-free (-□-) and "AZ" nutrient-enriched (-■-). Exact value is the average of 20 explants

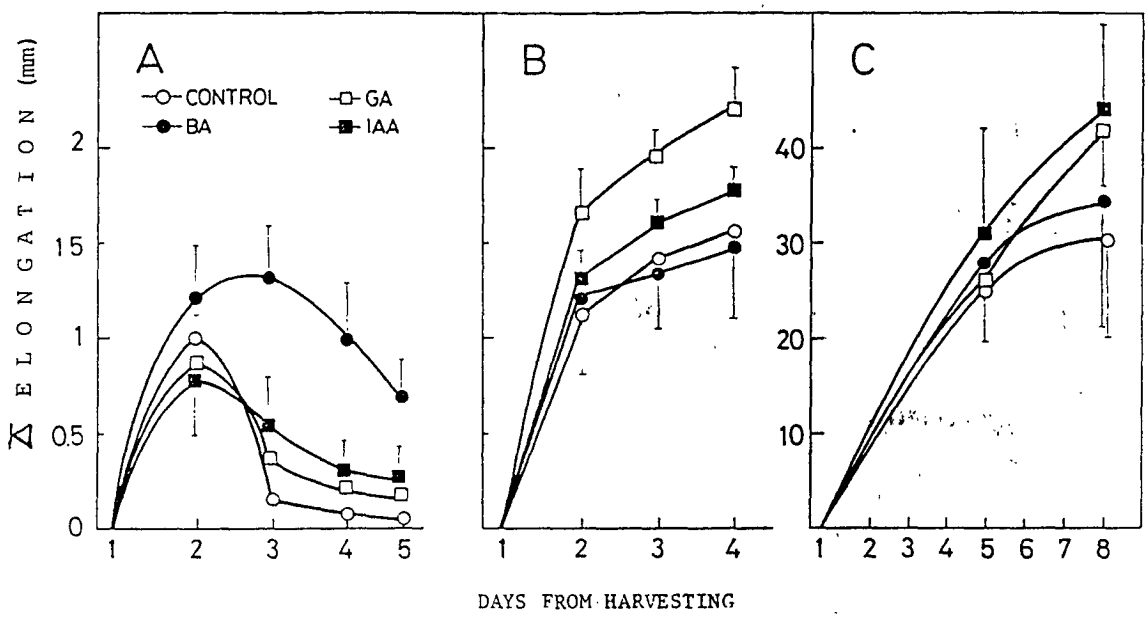

Figura 2: Effect of exogenous growth regulators + "AZ" enriched medium on the in vitro growth of sprout leaves during dormancy (A), end of dormancy (B), and sprouting time (C). Each value is the average of 20 explants.

control "AZ" culture medium, "A" + benzyladenine (BA) 10^{-6}, 10^{-7}, or 10^{-8}M; "AZ" + GA_3 10^{-9}, 10^{-10}, 10^{-11}, or 10^{-12}m; "AZ" + indol 3 acetic acid (IAA) 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}M, "AZ" + BA 10 M + GA_3 10^{-9} M + IAA 10^{-7} M + ABA 10^{-8} M

Experiment c. This experiment was done by placing sprout leaves over Wathman Pt 1 paper embebed with water (control) or with a methanolic extract of storage leaves

For all the experiments each treatment had one Petri dish with 20 explants for 20 culture tubes with one explant each, and treatments were repeated at least 3 times After an incubation time variable between 4 to 10 days (depending on the experiment), results were statistically analyzed at P = 0 05 level.

Growth regulator levels

Levels of GA-(both free-acid and glucosyl-conjugates), IAA-, and growth inhibitor-like substances, seed cloves were excised in the storage leaf and sprout leaf

These samples of the 3 sets, corresponding to different ontogenic status, were processed separately.

GA-Determination

The equivalent to 1 g d wt of sample was grounded in mortar with liquid nitrogen and 40 ml of aqueous 80% methanol (MeOH)

The mixture was filtrated throughout Wathman Pt 1 paper over Büchner funnel, and the residue extrated twice with extra 20 mL. of 80% MeOH. The filtrates were pooled and reduced to aqueous under vacuo, then adjusted to a final volumen of 60 mL with KH_2 PO_4 0 5 M pH 8.0. Aqueous was partitioned 5 times with half volumen of water saturated toluen. Then 50 mg of insoluble polyvinylpolypyrrolidone (PVPP, Sigma Chem Co) per mL of solvent were added to the aqueous phase and stirred 30 min. After filtration over Watman Pt 1 paper the PVPP was washed with extra 20 mL of KH_2 PO_4 buffer and then discarded. The aqueous phase was subsequently acidified to pH 2.5 with HCL 1 N and partitioned 4 times with water saturated ethyl acetate (EtOAc). The pooled EtOAc constituted the free acid EtOAc soluble fraction in which most of the free GAs would solubilize (with the exception of some polyhydroxilated GAs as GA_{32}). The remaining pH 2.5 aqueous phase was then partitioned 4 times with half volumen of water satured butanol (n-BuOH), and then discarded. The pooled n-BuOH would contain most of the glucosyl-conjugated GAs.

The volumen of both, EtOAc and n-BuOH fractions, were reduced under vacuo and an aliquot chromatographied onto Wathman lt 1 paper strips The chromatograms were developed15 cm ascendently with isopropanol. ammonia: water (100: 14: 6, v/v) in darkness The strips were dried with warm air and separated into 10 equal Rfs After elution with pure MeOH, each Rf was bioassayed by the dwarf rice

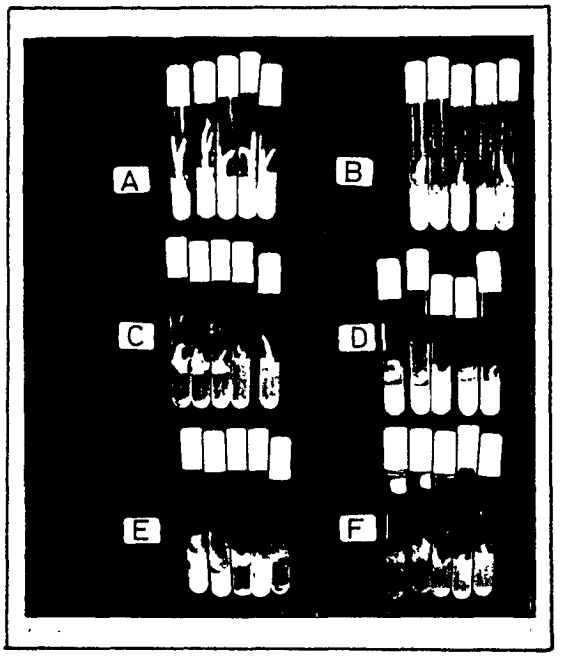

Figura 3: Effect of exogenous growth regulators on growth and differentation of isolates sprout leaves cultures in vitro during end of dormancy. A) GAS + BA + ABA; B) GAS, C) AIA; D) NA; E) Control; F) ABA.

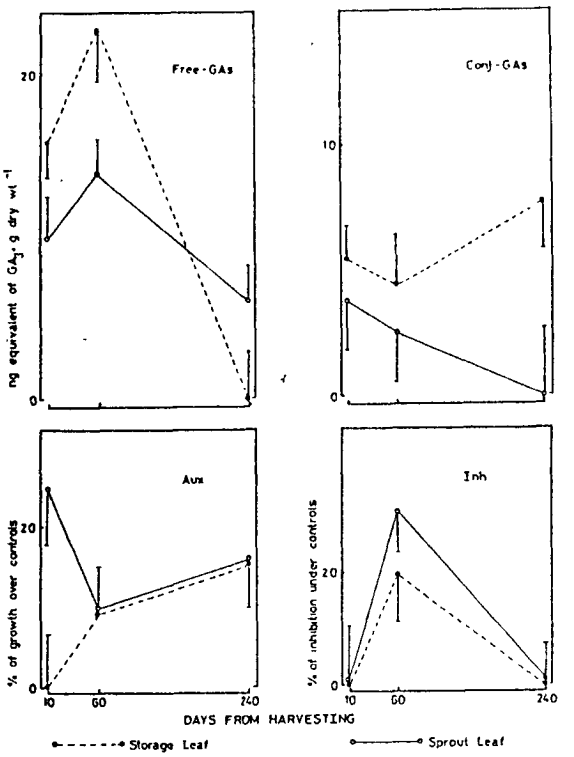

Figura 5: Changes in GA-like, auxin-like, and growth inhibitor-like bioactivity as measured by the u-drop and inmersion dwarf rice cv. Tanginbozu and the straight growth lenght wheat coleoptile bioassay

(*Oryza sativa* L) cv Tan-ginbozu test. For the EtOAc soluble free-acid GAs, the μ-drop version was employed (Murakami, 1968); while for the n-BuOH soluble glucosyl-conjugated GAs the inmersion procedure was used (Murakami, 1973). After measurement of the second leaf lenght. Results were statistically analyzed (P = 0.05) and the total activity found expressed as ng equivalent of GA_3 per g d wt of tissue

Auxin and growth inhibitor substances

Five g f wt of tissue were homogenized in a blendor with pure MeOH and allowed to stay overnigth at 4°C. The homogenate was filtered and washed with fresh MeOH. After reducing volumen to dryness under vacuo, the extract was retaken and partitioned with equal amounts of $NaHCO_3$ 0.1 M pH 8.0 and peroxide-free diethyl ether. The ether phase was discarded and the aqueous one acidified to pH 2.7 with HCL 1 N and re-extracted with diethyl ether.

The ether was reduced to dryness with warm air and anydrous Na_2SO_4. Then 1/5 aliquot of the extract was loaded onto 20 x 20 cm. Silica gel G UV_{254} (Macherey Nagel Co) 0 25 thickness plates After developing the chromatograms 10 cm with isopropanol: ammonia: water (100: 14: 6; v/v), Rfs and bands were carefully separated under UV light, eluted with pure MeOH and bioassayed by the straight lenght growth coleoptile test as previously described (Correa *et al*, 1975). Results were statistically analized (P = 0.05) and the total growth promoting activity above the control was expressed as auxin-like, while the coleoptile inhibition below that control was considered as growth inhibitor-like.

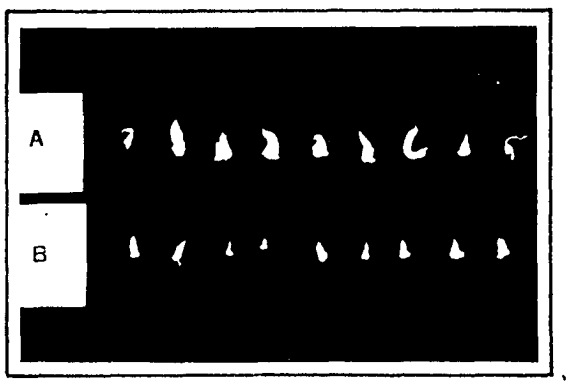

Figura 4: Isolated garlic sprout leaves during dormancy, after incubation with (A) and without (B) extracts of storage leaf at the end of dormancy

RESULTS

Figure 1 shows the *in vitro* growth rate of isolated sprout leaves during dormant and sprouting periods, either on semi-solid nutrient free-agar medium or "AZ" nutrient enriched semi-solid culture-medium The growth rate for dormant sprout leaves was higher in those put onto the "AZ" enriched medium and the same occurred for the sprouting ones However the latter grew more rapidly than those in dormant condition, wichever the medium used.

The effect of the different growth regulators over the *in vitro* growth rate of isolated sprout leaves in the three ontogenic conditions are showed in Figure 2 (only one concentration for each regulator). During dormancy BA 10^{-8} M produced a significative response, while GA_3 10^{-9} M was more effective towards the end of dormancy. No response was found with any of the growth substances in all the concentrations assayed for sprouting time; IAA and abscisic acid (ABA) (data not showed) did not show significative differences with control in any case.

When all the growth substances (including ABA) were assayed together onto the isolated sprout leaves cultured *in vitro* at the end of dormancy (Figure 3), there was a noticeable increase in the growth rate and differentiation of the second and third leaves, in contrast with control and growth regulators treatments alone.

Figure 4 shows the results with dormant sprout leaves cultures in Petri dishes with and without storage leaf; increases in size was obtained in explants growing alone but with an storage leaf extract.

Growth regulator levels found are showed in Figure 5. Free acid GA-like substances increased towards the end of dormancy in both, sprout and storage leaves; the higher activity being found in the latter. Glucosyl-conjugate GA-like substances were mostly decreasing in sprout leaft over the time after harvest, although in the storage leaf at sprouting time an increase occurred. Auxin-like substances showed to be rather high during dormancy in sprout leaf, there after became similar to levels found in storage leaf. Growth inhibitor-like substances showed a behaviour quite similar to free GAs.

DISCUSSION

The results obtained in this work show that isolated sprout leaves cultures *in vitro* can grow even if they are dormant (Figure 1). This suggests that the physiological control of dormancy can be located, at least partially in the storage leaf.

This agrees with the statment of Kothari (1979), who suggested that callose deposit obturates phloem vessels in garlic storage leaves thus being the main factor in determining dormancy, and dissapears before sprouting The same does not happen in *Allium vinale* (Stritzke and Peters, 1970) in which control of dormancy is located in the sprout leaf. The difference can be explained by the fact that wild specie belongs to a very different evolutive step.

By isolating the sprout leaf, growth rate under *in vitro* culture conditions depended on its ontogenic status (dormant or sprouting), and also of some nutritive factors. This implies that just after harvest sprout leaf does not posses a complete set of hormonal signals and nutritive compounds which would allow it to grow. And those chemical signals and trophic factors might be dependent of the release from the storage leaf.

Taking in to account the response of isolated sprout leaves to different exogenous growth regulators, a possible involvement of endogenous cytokinins can be stated in the dormant period. This dormant period has been characterized by an active differentiation (Argüello, 1987), thus a possible role for this kind of hormone seems reasonable since Rhakimbaev and Solomina (1980) reported cytokinin-like activity during garlic dormany.

Albeit no response was obteined with IAA application, the participation of auxins in this ontogenetic step can not be rouled out, specially being in mind the relatively high levels of auxin-like activity found in the dormant sprout leaves (Figure 5), wich could be enough by themselves, or with cytokinins to provoke cell division/differentiation

At the end of dormancy explants showed responses to GA_3 (Figure 2) in coincidence with higher levels of GA-like activity found in both, storage and sprout leaves. This fact, indicative of an active GA turnover, is coincidental with similar findings in potato (A. de Bottini *et al.*, 1982) and peach flower buds (Luna *et al.*, 1990).

The higher levels of growth inhibitor-like substances found by that time for both sprout and storage leaves, can be indicative of an active metabolism which prepares tissues to sprout, but at the same time do not allow them to grow

During sprouting, sprout leaf did not show responsive to any regulator added, eventhough general levels of growth substances were low (except perhaps for auxins). This situation can imply that tissues are already triggered towards a sensitive condition to grow, and a low level of endogenous hormones is enough.

Differences in the response of the explants to exogenous growth regulators can be due to a different degree of differentiation reached during the post-harvest, which in turn might mean a diverse-sensitiveness of target tissues to the chemical signals. The evidence found allows to state that storage leaf can control differentiation and growth of sprout leaf, either troughout nutritive factors or chemical signals, or both.

Some previous reports with whole garlic bulbs (Rhakimbaev and Ol'Shanskaya, 1976) proposed that conjugated GAs are characteristic of the dormant state, with no presence of free GAs by that time Our results are rather different in the sense that they showed high levels of free GAs during dormancy, specially at the end of dormancy and in the storage leaf, which in turn suggests this high levels might be important in the mobilization of sacharids towards the sprout leaf. This correlates well with a pronunced decrease in the soluble carbohydrates content of the storage leaf (Argüello, 1987).

Notwithstanding, not only hormonal and nutritive factors might regulate the dormancy/sprouting processes, since a physicomechanical effect (like gas diffusion impediment) of storage leaf can not be rouled out Our results however, do not agree with Lewak's proposal since simultaneous hormonal control seems to operate.

Finally, it can be stated that there is a certain dependence of sprout leaf respect to the storage one, wich in some way partially controls garlic seed cloves dormant status. This dependence is more tight during dormancy, and becom looser as post-harvest time goes by. Thus, sprout leaf would underpass several steps of differentiation (wich depend on hormonal, nutritional, and physico-mechanical influences from the storage leaf) before sprouting.

ACKNOWLEDGMENTS

Supported with funds provided by Consejo de Investigaciones Científicas y Tecnológicas de la Provincia de Córdoba. (CONICOR) and CAFTA (Comisión Administradora Fondo Promoción Tecnología Agropecuaria). R.A.

BIBLIOGRAPHY

Abo-El Nil, M.M. and F.W. Zettler, 1976. Callus initiation and organ differentiation from shoot tip cultures of *Calocasia esculenta*. Plant Sci Letters 6, 401.

A de Bottini, G., R. Bottini and R. Tizio, 1982 Physiology of dormancy in potato tubers as related to levels of endogenous regulators. Phyton 42, 115.

Altman, A. and R. Goren, 1974. Growth and dormancy cycles in citrus bud cultures and their hormonal control Physiol Plant 30, 240.

Argüello, J A., R. Bottini, R Luna, G. A de Bottini and R.W. Racca, 1983 Dormancy in garlic (*Allium sativum* L.) cv Rosado Paraguayo.

Levels of growth substances in "seed cloves" under storage. Plant Cell Physiol 24 1559

Argüello, J A., 1987 Fisiología de Post-Cosecha en ajo (*Allium sativum* L) Contribución al estudio del mecanismo de la dormición y la brotación. Tesis doctoral. Universidad Nacional del Sur (Argentina).

Borkawska, B. and L.E. Powel, 1979. The dormancy status of apple buds as determined by in vitro culture systems· J Amer Soc. Hort Sci 104, 708.

Correa, N S., R. Bottini, G. A. de Bottini, L. Goleniwski and L. Gordon, 1975. Cambios en los niveles de inhibidores del crecimiento y de giberelinas endógenas durante el reposo vegetativo de yemas de duraznero. Phyton 33. 193.

Goodwing, P.B., 1983. In: The growth of the potato. Ivins, J.D., Milthorpe, F.L. eds, pp 63. Butterworths, London.

Kothari, I.L., 1979. Morphohystogenic and anatomical studies in garlic: Phloem. Proc Indian Acad Sci 88. 219.

Lewak, S., 1979. Hormonal Regulation of dormancy in plants. Acta Horticultural 91. 41.

Luna, V., E. Lorenzo, H. Reinoso, M. Tordable, G. Abdala, R.P. Pharis and R. Bottini, 1990. Dormancy in peach (*Prunus Persica* L.) flower buds. I. Floral morphogenesis and endogenous gibberellins at the end of the dormancy period. Plant Physiol 93, 20.

Mahotiere, S. R.C. Herner, F G. Dennis, 1976. Effects of applied growth substances on growth of shoots apices excised from onions in rest. Physiol Plant 110, 211.

Murakami, Y., 1968. New rice seedling bioassay for gibberellins "Microdrop Method" and its use for testing extracts of rice and morning glory. Not Mag 81, 33.

Murakami, Y., 1973. A method for detecting bound gibberellins by rice seedling test. Chem Reg Plants G. 40.

Naver, E.M. and S.B. Boswell, 1981. Stimulating growth of quiescent citrus buds with 6-benzylaminopurine. Hort Sci 16 162.

Phillips, I.D.J., 1962. Some interactions of gibberellic acid with naringenin (5, 7, 4 tri-hidroxi flavonone) in the control of dormancy and growth in plants, J Exptl Bot 13, 213.

Rakhimbaev, I.R.; Ol'Shamskaya., 1976. Dinamics of endrogenous gibberellins during the process of transition of garlic from the state of dormancy to active growth. Soviet Plant Physiol 23, 76.

Rakhimbaev, I.R. and V.F. Solomina, 1980. The activity of endogenous cytokinins during garlic storage at low temperature. Vestnik Sel Khoz Nauki Kazakhstana 2, 46.

Rappaport, L., M.D. Blumenthal-Goldschmith and O.E. Smith, 1965. Regulation of bud rest in tubers of Solanum tuberosum L. I. Effect of growth substances on excised potato buds. Plant Cell Physiol 6, 587.

Sanchez, R.A. and L. De Miguel, 1983. Aging of *Datura ferox* seed embryos under dry storage and its reversal during inhibition. Z. Pflanzephysiol 110. 319.

Shibakusa, R., 1979. Studies on the dormancy of *Abbies sanchalimensis* Masters. (IV). Effects of growth substances on the growth of cultures embryonic shoots in the dormant period. Jap Forestry Soc. 61, 47.

Stow, J.R., 1976. The effects of defoliation on storage potential of bulbs of the onion (*Allium cepa*). Ann Appl Biol 84, 71

Stritzke, J.E and E.J. Peters, 1970. Dormancy and sprouting cycles of wild garlic. Weed Sci 18, 122.

Thomas, T H , 1969 The role of growth substances in the regulation of onion bulb dormancy. J. Exptl Bot 20, 124.

Thomas, T.H., 1981 Hormonal control of dormancy in relation to post-harvest horticulture. Ann Appl Biol 98, 525.

Tizio, R., 1982. Fisiología de la dormición en tubérculos de papa y sus relaciones con el mecanismo hormonal de la tuberización Rev Cs Agropecuarias, III.'91.

Weinberger, J H., 1969 The simulation of dormant peach buds by a cytokinin Hort Sci 4, 125