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Biochemical Changes in *Pinus pinea* Seeds. II. The Stimulation of Isocitrate Lyase and Malate Dehydrogenase Activities by Exogenous Growth Substances

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Treatment of intact pine seeds with plant hormones and steroid substances for several days, after an initial 24 hour immersion in different hormone solutions, resulted in a substantial increase in the specific activity of isocitrate lyase and malate dehydrogenase than the observed in the corresponding control solutions. Fresh weights did not show any increase, because water uptake was reduced in presence of plant hormones (indolacetic acid, kinetine and giberellic acid). However, dry weights were consistently greater than those of the control solutions.

Pine seeds *(P. pinea)* can be induced to germinate by stratification (11) or by application of growth substances (16).

Isocitrate lyase (E.C.4.1.3.1.) and malate dehydrogenase (E.C.1.1.1.3.7.) were present in the megagametophyte of dry pine seeds. The activity of these enzymes markedly increased during germination, or by a chilling treatment, which appears to activate the mechanism for plant hormones synthesis, in several types of seeds (11). As germination of *Pinus pinea* seeds requires a mobilization of the lipid reserves stored in the megagametophyte, an investigation of the effects of kinetine (K), indolacetic acid (IAA), giberellic acid (GA_3) and steroid hormones; testosterone (T), estrone (E) and estradiol (EL), on the activities of those enzymes considered to participate in the conversion of fat to sucrose has been undertaken. The objetives of this study, were the determination of changes in levels of isocitrate lyase (IL) and malate dehydrogenase (MDH) during germination in megagametophyte of *Pinus pinea* seeds, by addition of exogenous growth substances to the growth medium.

Materials and Methods

Pine seeds (*Pinus pinea* from Coca, 1 year old) were used throughout this work. Treatment of seeds with either growth substances or water was carried out as described by PINFIELD (20). The seeds surface was sterilised in sodium hypochlorite solution (1 % available chlorine) for 10 minutes, immersed in sterile distilled water for 1 hour and then in hormones solutions or distilled water for 24 hours. Subsequently, they were transferred to petri dishes with vermiculite for germination. Every day, the seeds (50 in each dish) were moistened either with 50 ml of hormone solution or water.

Samples of seeds were taken from each series at intervals during the following 12 days for determination of fresh weight, dry weight and IL and MDH activity in the megagametophytes.

The enzymes were extracted with 0.1 M potassium phosphatase buffer (*p*H 7.6) for 10 minutes in a mortar. The homogenates were filtered through muslin and the resulting slurry was centrifuged for 10 minutes at 10,000 g at 0-2° C. The supernatant fluids were used for IL and MDH assays.

IL was assayed by the methods of DIXON and KORNBERG (2), at *p*H 7.9 and 30° C. MDH was determined by the BERGMEYER and BERNT method (1), at *p*H 7.4 and 25° C. Final volume for IL and MDH assays was 1 ml; it contained 10-100 μl of enzimatic extract.

Protein content was determined by the method of LOWRY and ROSENBROUGH (13).

Results and Discussion

The germinative behaviour of *P. pinea* seeds is shown in figures 1 and 2. Maximum germination, as measured by protusion of the radicle, was attained after 40 days of hormonal treatment. During the first 5-8 days, no germination was recorded in IAA, K, GA_3 and T, EL, E

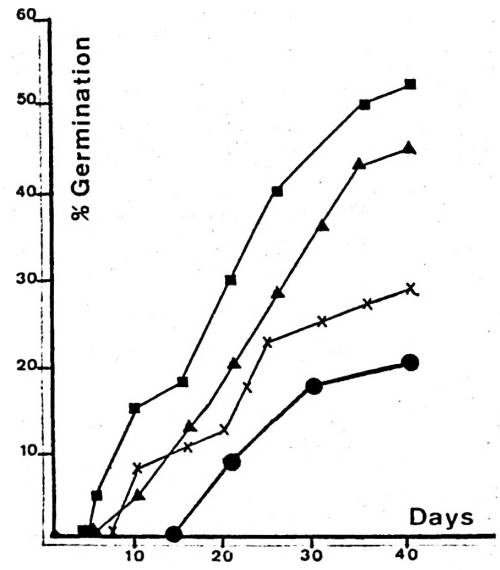

Fig. 1. *Germination of pine seeds of Coca 1 year old at 25-26° C.*
(■) Kinetine (0.1 mg/l); (×) Indolacetic acid (0.1 mg/l); (▲) Giberellic acid (0.1 mg/l); (●) control.

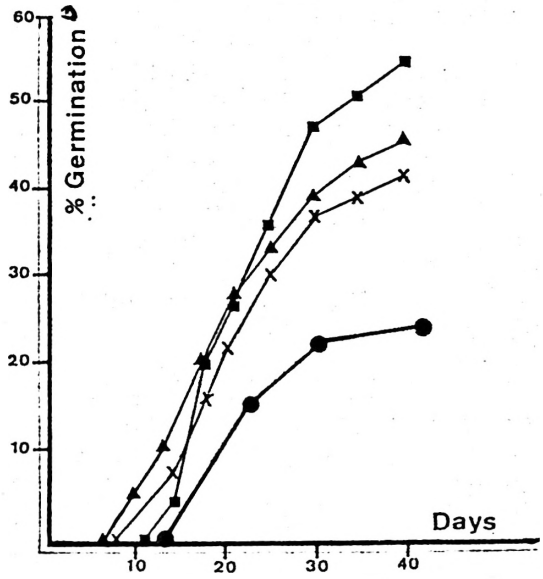

Fig. 2. *Germination of pine seeds of Coca 1 year old at 25-26° C.*
(▲) Testosterone (1 mg/); (■) Estradiol (10 mg/l); (×) Estrone (10 mg/l); (●) Control.

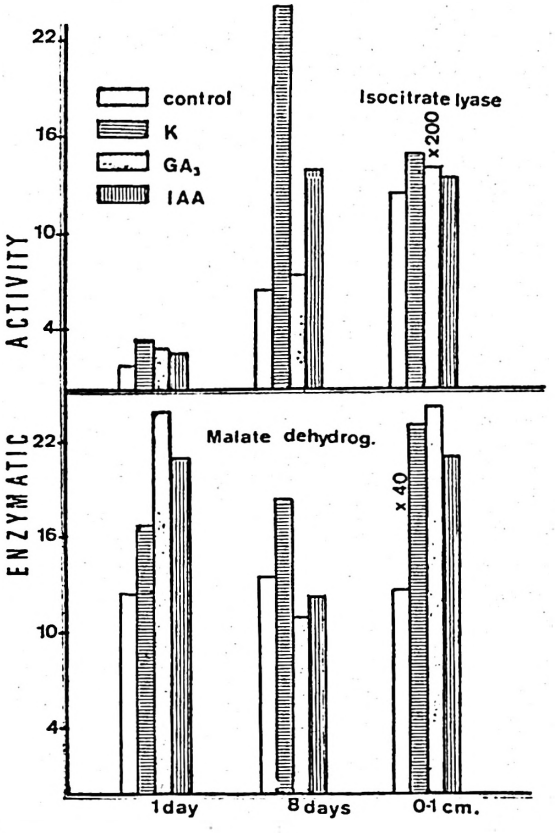

Fig. 3. *Changes of specific activity of isocitrate lyase and malate dehydrogenase, in the endosperm of pine seeds.*
IL and MDH was expressed as μmoles of substrate converted per minute, per mg of protein. Germination time 1, 8 days after seeds were sown and seedlings with 0-1 cm radicle.

respectively, which indicates that the observed changes in IL and MDH activities (fig. 3 and 5) preceded radicle protusion.

The megagametophytes of dormant pine seeds showed very low levels of IL and MDH activities, but the immersion of seeds for 24 hours in different solutions markedly increased the specific activity.

The presence of K, GA_3 and IAA in the culture solution induced a larger increase in IL activity than in water control (fig. 3). The maximal differences were observed in endosperm from 8 days old seeds, where K showed a 6-fold increase. MDH activity is also enhanced when K, IAA and GA_3 are present in the control solution.

PENNER and ASHTON (19) have observed that the presence of benzyladenine, a cytokinin, in the culture solution can replace, in part, the hormonal stimulus, produced by the embryonic axis, which is normally required for maximum development of IL activity in cotyledons (reserve tissue).

IL has been proved to be «de-novo» synthesized in cotyledons of germinating peanuts (3, 12). The effects of GA_3 on IL should be, to some extent, analogous to the effects of GA_3 on the aleurone layer

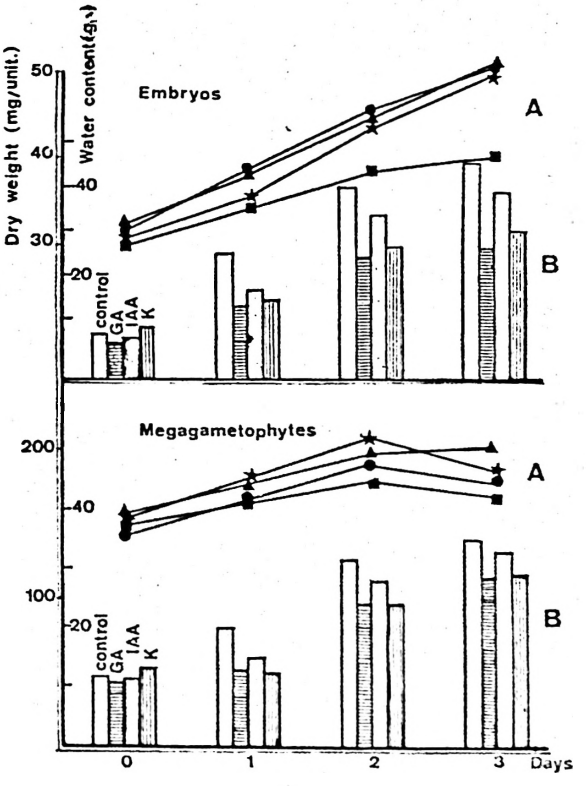

Fig. 4. *Effects of Giberellic acid, Kinetine and Indolacetic acid on the dry weight (A) and water content (B).*
●—● GA_3; ▲—▲ K; ★—★ IAA; ■—■ water control.

of barley, in which gibberelin has been shown to induce a de-novo synthesis of α-amylase VARNER and CHANDRA (22) and PALEG (18).

The fresh weights of embryos and endosperm did not show any increase when seeds were treated with K, IAA and GA_3; however, the dry weights were consistently greater than those of the water controls. Fresh weights did not increase, because water uptake was reduced in presence of plant hormones (fig. 4). A similar effect was reported in other materials by HEINRICH (5, 6) and POHL (21), who observed changes in water permeability.

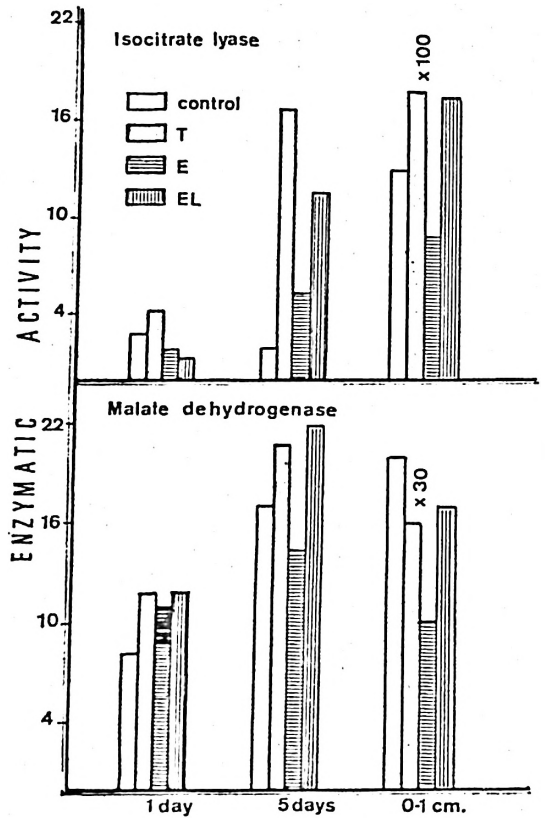

Fig. 5. *Changes of specific activity of isocitrate lyase and malate dehydrogenase in the endosperm of pine seeds.*

IL and MDH was expressed as μmoles of substrate converted per minute per mg of protein. Germination time 1, 5 days after seeds were sown and seedling with 0-1 cm radicle.

The second type of substances T, EL and E studied have a similar action on the levels of IL and MDH activity. It has been observed the greatest changes in 5-day-old seeds after adding the hormones to the germination medium.

The best responses were obtained when seeds were treated with either T or EL. They show an increase about 6-9 fold in IL activity (fig. 5). The action of this substances could be explained by an influence of steroid hormones with a simultaneous increase of endogeneous gibberellins, auxin and kinetin, as it was observed by KOPCEWICZ (7, 8, 9) in several plants and seeds.

Fresh weights and dry weights were not apreciably greater after exposure to steroid hormones than after treatment with sterile distilled water (1 % tween).

Although it is known that light is a very important factor, necessary for the correct course of pine and others seeds germination, and gibberellins and cytokinins may partly of fully satisfy this requirement, as it has been shown by KOPCEWICZ (10) in pine seeds, we have been unable to find this effect. Slight changes were only observed.

On this way, other series of experiments showed the effect of K and T on IL and MDH activities, when germination was taking place either in light or in darkness.

Similar effect of K and T were obtained in this study, but enzimatic activities levels were lower in darkness than in light (table I).

These results show a possible interaction between light and chemical treatment with hormonal substances. Relationships of kinetin and light were also observed by GRZESIUK and REJOWSKI (4), MILLER (14, 15) and NIKOLAJEWA (17).

Table I. *Influence of growth regulators, on the leveles of IL and MDH activities in pine megagamethophytes.*
Enzymatic activity was expressed as μmoles of substrate converted per minute per mg of protein. Numbers between brackets were the results expressed as percentage (%) of control.

CULTURE SOLUTIONS	Days after seeds were sown				
	0	3		5	
		light	dark	light	dark
Isocitrate lyase					
Water	52.60 (100.00)	56.26 (107.00)	42.60 (80.98)	117.30 (223.03)	93.80 (178.71)
Kinetine	50.68 (96.34)	80.07 (152.49)	64.69 (122.98)	235.17 (447.86)	134.48 (255.66)
Tween	29.86 (56.76)	40.24 (76.50)	52.22 (99.27)	82.50 (156.84)	79.66 (151.44)
Testosterone	43.42 (82.54)	77.81 (147.92)	57.14 (108.63)	148.99 (283.25)	132.81 (252.43)
Malate dehydrogenase					
Water	30.40 (100.00)	40.83 (130.03)	34.93 (114.90)	28.26 (92.96)	19.29 (63.45)
Kinetine	34.11 (112.20)	46.28 (152.23)	37.75 (124.17)	60.05 (197.53)	42.64 (140.46)
Tween	39.09 (128.59)	26.31 (86.54)	22.38 (73.62)	51.13 (168.19)	40.34 (132.70)
Testosterone	43.73 (143.85)	37.19 (122.34)	35.12 (115.52)	56.62 (186.23)	43.36 (142.30)

Resumen

El tratamiento de semillas intactas de pino con hormonas vegetales y sustancias de carácter esteroídico por varios días, después de 24 horas de inmersión en las distintas soluciones hormonales, produce un incremento en IL y MDH, mayor al observado en las soluciones controles.

El peso fresco no muestra ningún incremento, porque la absorción de agua se encuentra reducida en presencia de las hormonas vegetales (ácido indolacético, kinetina, ácido giberélico). Sin embargo, los valores de peso seco son mayores que los que su control.

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