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Physiological studies on dormancy in grape seeds (Vitis Vinifera)

1. On the naturally occurring growth substances in grape seeds and their changes during low temperature after ripening

by

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Introduction

Grape seeds require a period of three months of moist after-ripening at 5° C for germination, (Flemion 1937, Singh 1961, Randhawa and Negi 1964). Certain chemicals such as gibberellic acid and thiourea have also been found effective in partially substituting this after-ripening period there by hastening seed germination (Fox and Tellechea 1957, Randhawa and Negi 1964, Chadha 1965). But the factors responsible for the dormancy in grape seeds and the physiological changes taking place during low temperature stratification leading to germination have not been studied so far. However, recent studies on seeds and buds of a number of plants (Luckwill 1952, Hemberg 1965, Vegis 1965, Wareing 1965, Thomas *et al.* 1965) have demonstrated the involvement of certain growth inhibiting and growth promoting substances in the dormancy mechanism found in such organs.

Investigations of this type would elucidate the factors involved in the physiology of dormancy in grape seeds. The results of our studies conducted during 1966-67 on the naturally occurring growth substances in dormancy of grape seeds, their changes during moist after-ripening at 5° C and dry storage at room temperature are discussed in this report.

Materials and Methods

Grape seeds were collected from fully ripe berries of the variety Bharat Early, grown at the Experimental Orchard of Indian Agricultural Research Institute. The seeds, after cleaning, were surface dried and were divided into two lots. One lot was mixed with moist sand in a petri dish and was stored in a refrigerator at 5° C. The other lot was stored dry at room temperature.

The germination of the fresh seeds was tested immediately after extraction and that of chilled and non-chilled seeds later at monthly intervals. Germination tests were carried out by sowing seeds in sterilized sand and kept at a temperature of 25—28° C. When taking seed samples for germination tests, a duplicate lot was taken from both the treatments for extracting the endogenous growth substances.

Extraction of growth substances

Seeds were macerated in a chilled mortar with cold methanol. The macerated material was submerged in an excess of methanol and allowed to stand at —23° C

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for 20 hours with occasional stirring. At the end of the extraction period, the methanolic extract was filtered and the residue was washed with fresh methanol. These washings were added to the original filtrate and the whole extract was evaporated to dryness in a Buchler model flash evaporator under reduced pressure at 35—40° C. The dry residue was dissolved in a known volume of methanol for subsequent purification and bioassay.

For determining the total auxins and inhibitors, an aliquot of the above methanolic extract was evaporated to dryness and redissolved in acetonitrile which was shaken with an equal volume of hexane (NITSCH 1956). The acetonitrile phase, after evaporating to dryness, was dissolved in a known volume of methanol for chromatography.

The remaining part of the original concentrated methanolic extract corresponding to a known weight of seeds was evaporated to dryness. The residue was dissolved in distilled water. The acidic gibberellin-like substances were extracted from this aqueous solution using ethylacetate by the method suggested by KATO (1964).

Paper Chromatography of growth substances

Descending paper chromatography in darkness at a temperature of 25° C ± 2 using Whatman No. 1 chromatographic filter paper was done throughout this study. An aliquot of the final methanolic solution containing growth substances corresponding to a known weight of the seed sample to be tested was applied along a five cm starting line of the paper. The chromatograms were equilibrated for 6—8 hours in the solvent vapour and then developed in isopropanol/ammonia/water (10/1/1 : v/v/v) to a distance of 30 cm. Occasionally, chromatograms of seed extracts were developed in distilled water and 70% ethyl alcohol along with synthetic IAA. The developed chromatograms were sprayed with Ehrlich and Salkowski reagents (KEFFORD 1955) for locating the growth substances and their possible identification.

Bioassay: The auxin activity in the developed chromatograms was tested using *Avena* (var. Algerian) first internode test as suggested by NITSCH and NITSCH (1956). All tests were conducted in a temperature controlled dark room (25° C ± 2) with subdued green light. Final measurements of the sections were made using a photographic enlarger and recording the magnified (×5) images with a mm-scale. Statistical analysis was done to show significant differences. The response of first internode sections of the variety Algerian to synthetic IAA (0.0001—1.0 μg) and GA_3 (0.001—100 μg) was also tested along with the seed extracts.

Inhibitors: The presence and the activity of the inhibitors present in the extracts of grape seeds were studied by cress *(Lepidium sativum)* seed germination test. The chromatograms of seed extracts were cut into 10 equal pieces and were placed in 5 cm petri-dishes and moistened with two ml of double distilled water. As control, a blank filter paper strip was used in the petri-dish. In each of these petri-dishes, 50 cress seeds were allowed to germinate in darkness at a temperature of 25° C ± 2. The number of seeds germinated was counted after 48 hours and the percentage inhibition of germination was calculated from duplicate tests.

The chromatograms presumably containing gibberellin-like substances were dried and cut into equal parts, shredded and eluted with 10 ml of 80% alcohol. The resulting eluates were dried under a current of air and redissolved in a known volume of 0.05 per cent Tween 80 solution. Aliquots of this were tested for biological activity using the dwarf maize assay, (D_5 dwarf mutant maize plants; PHINNEY 1961).

Results

The germination percentage of fresh seeds as well as those stored at 5° C and room temperature for different periods are given in Table 1.

Table 1

Germination percentage of grape seeds (var. Bharat Early) stored at 5° C and room temperature for different periods

Period of storage	Germination percentage	
	5° C	Room temperature
Fresh	—	—
1 month	10	—
2 months	26	6
3 months	60	18

It is evident from the above data that freshly harvested grape seeds were in a state of dormancy and did not germinate. This state of dormancy continued in the seeds stored at room temperature during the first month and even after the second and third month of storage, only a meager percentage of germination such as 6 and 18 respectively was recorded. This indicates that dormancy in grape seeds is not broken during dry storage at room temperature. On the contrary, seeds stored in moist sand at 5° C germinated to the extent of 26 per cent after two months and 60 per cent after 3 months storage, indicating a gradual breakage of dormancy.

The results of the bioassay for auxins in the extracts of fresh, non-chilled and chilled seeds (corresponding to 0.5 g of seed) during different months of storage are presented in Figure 1. It can be seen from the histograms in Figure 1 that in the chromatograms of fresh seed extracts, growth activity was located in two zones at Rf 0.3—0.5 and Rf 0.8. The peak activity was at 0.3—0.5. In the chromatograms of seed extracts taken after one, two and three months of storage at 5° C, the activity of this zone was diffused over Rf 0.0—0.6 which may be due to the high amount of growth active substance present in such extracts. The amount of growth active substances present at Rf 0.0—0.6 in each chromatogram was calculated in terms of IAA equivalents and is presented in Table 2.

Table 2

Auxin level in the extracts of chilled and non-chilled grape seeds var. Bharat Early. Results expressed as IAA equivalents in μg at Rf 0.0 — 0.6 of the chromatograms representing 0.5 g of seeds

Storage period	Chilled	Non-chilled
Fresh	—	0.004
1 month	0.126	0.0035
2 months	0.469	0.0028
3 months	0.569	0.189

It is evident from Figure 1 and Table 2 that the extracts of chilled and non-chilled seeds showed a marked difference in the levels of growth promoting substances during storage. The extracts of fresh seeds contained only 0.004 μg IAA equivalent, whereas a gradual and steady increase in the amount of such growth

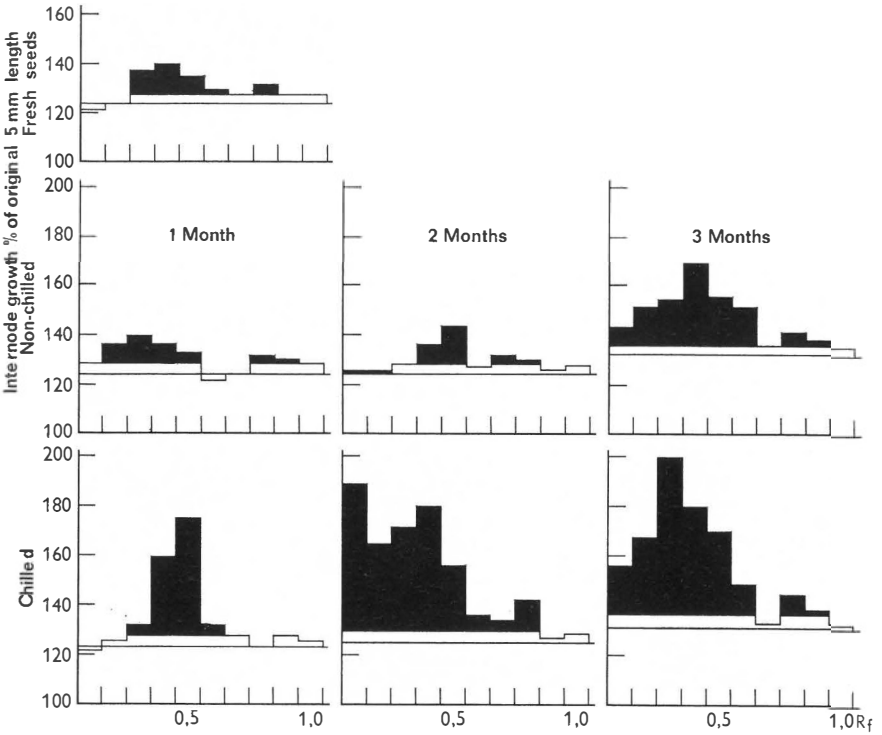

Fig. 1: Acetonitrile soluble growth promoting substances in the extracts corresponding to 0.5 g of chilled and non-chilled grape seeds (variety Bharat-Early). Darkened areas represent significant growth at 5 percent level.

promoting substances was noticed in the extracts of chilled seeds during storage. In the extracts of seeds stored at room temperature, a slight increase in the amount of growth promoting substances was noticed only after 3 months of storage which was however insignificant when compared to that of chilled seeds. The increase in the germination percentage of seeds stored at 5° C was thus correlated with an increase in the amount of growth active substances in the seeds.

When the developed chromatogram of extracts of seeds stored for three months at 5° C was sprayed with Ehrlich reagent, a bright pink colour turning blue after heating was developed at Rf 0.2—0.4. The intensity of this colour development, even though observed in the chromatograms of seed extracts stored at 5° C and room temperature for one and two months, was much less in the latter case than of seed extracts stored at 5° C for three months. Synthetic IAA located at Rf 0.4—0.5 when developed, also gave a pink colour turning blue after heating. However, colour development with Salkowski reagent, which is a more specific test for indole auxins, was not observed in the chromatograms of chilled or non-chilled grape seed extracts. Also, chromatograms of seed extracts developed in 70 per cent ethanol and distilled water did not give any conclusive proof to establish the identity of the growth active substance as IAA.

The chromatograms of fresh, chilled and non-chilled seed extracts taken during different periods of storage although inhibiting the germination of cress seeds at

Rf 0.7—0.8, indicating the presence of inhibitors, no difference was noticed in the degree of inhibition of cress seed germination by different seed extracts.

Bioassay of the acidic ethylacetate fraction of the seed extracts on D_5 dwarf maize mutant plants did not show any growth response in the first and second leaf sheath elongation.

Discussion

A close correlation between an increase in the amount of growth promoting activity in the extracts of grape seeds during stratification and the removal of dormancy was noticed in the present study. The extracts of freshly harvested seeds as well as those stored dry at room temperature for three months contained much smaller amounts of such growth promoting substances as compared to the seeds stratified for three months. LUCKWILL (1952), in apple seeds, also found that low temperature moist after-ripening resulted in an increase in the growth promoting activity of seed extracts. Such stratification treatment also reduced the amount of an inhibitor present in apple seeds. Thus, LUCKWILL concluded that the synthesis of growth promoting substances coupled with the leaching out of the inhibitor from the seeds during low temperature after-ripening resulted in the germination of apple seeds. The presence of growth inhibiting substances in grape seeds has been reported by LUCKWILL (1953). In the present study, no correlation between dormancy and the level of such inhibitor was observed, although inhibition of cress seed germination was noticed in the chromatogram of seed extracts at Rf 0.7—0.8. WAREING (1965) has given evidence to show that growth inhibitors are involved in the regulation of dormancy in many seeds, although in seeds like pea nut, GORDEN-SHARIR and WAREING (1964) could not isolate any inhibitor from dormant seeds. Thus, the presence of inhibitors alone cannot be taken as the sole factor controlling dormancy in seeds. PILLAY and EDGERTON (1965) reported an increased level of growth promoting substances in the extracts of after-ripened cherry seeds with and without gibberellic acid pre-treatment. The present findings also show that stratification resulted in increased synthesis of growth promoting substances which may be necessary for the germination of grape seeds.

The importance of growth promoting and gibberellin-like substances in the dormancy regulation of seeds has been stressed by a number of workers especially during the onset of dormancy, (VILLERS and WAREING 1960, FRANKLAND and WAREING 1962, WAREING 1965). In our experiments, the presence of gibberellin-like substances could not be detected in D_5 dwarf maize mutant bioassay. Since oat first internodes from the variety Algerian do not respond to gibberellin-like substances significantly, it is to be concluded that this large amount of growth promotion obtained at Rf 0.0—0.6 in the chromatograms of chilled seed extracts is due to substances similar to auxins. However, none of the chromogenic or chromatographic tests indicated that this substance is identical with IAA. Also, when the chromatograms of chilled seed extracts were developed in distilled water and 70 per cent ethanol, growth activity was located at Rf 0.4—0.5, 0.9—1.0; and 0.7—0.8 respectively. Hence it is to be assumed that a complex of substances with growth promoting activity are synthesised in the grape seeds during low temperature storage.

Since GA_3 has been found effective in hastening the breakage of dormancy in grape seeds (RANDHAWA and NEGI, 1964), it is reasonable to assume that low temperature after-ripening results in an increased synthesis of endogenous gibberellins in the seeds. However, our results do not show any evidence to substantiate this.

Hence, further experiments are under way to see how GA_3 treatment brings about enhanced germination of partially chilled grape seeds. The results of our studies in regard to the effect of GA_3 on the metabolism of auxin-like growth promoting substances present in grape seeds and their exact biological and chemical properties will be communicated at a later date.

Summary

In order to understand the hormonal factors affecting dormancy in grape seeds, studies were conducted on the endogenous auxins, inhibitors and gibberellin-like substances in the seeds stored moist at 5° C and at room temperature .The results show that moist after-ripening at 5° C resulted in an increase in the amount of growth promoting substances similar to auxins, in the extracts of chilled seeds. No correlation was observed between the content of inhibitor and dormancy in grape seeds. The extracts of neither chilled nor non-chilled seeds showed the presence of gibberellin-like substances when tested on D_5 dwarf maize mutant plants.

In the light of these findings, it is proposed that an increased synthesis of growth promoting substances during stratification may be one of the factors responsible for the termination of dormancy and resumption of germination of grape seeds.

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