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Influence of Cold Stratification and Germination on Saponins in *Pinus pinea* Seeds

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Saponin determination were made on several types of seeds: non-stratified, stratified on wetted vermiculite at 4° C during several periods, seeds on germination in a Jacobsen chamber at 29° C and on seedlings with radicle lengths ranging from 1 to 130 mm.

The highest levels of saponin increase was reached on the 4th day of stratification, followed by another increase after values decreased afterwards but always remained higher then those obtained before initiation of stratification.

When the seedling radicles reached lengths of 5-15 mm, and lengths of 100-130 mm, germination produced significant increases of up to 60.86 mg/100 g of dry weight. Soaking *Pinus pinea* seeds in strong concentrations of saponin produced a decrease in the germination percentage. At low concentrations, howewer, it stimulated germination.

Our Department has been working on the metabolic changes of the more important constituents of *Pinus pinea* seeds, subjected to cold stratification and germination (8-12).

Aqueous extracts from defatted seeds, showed after vigorous shaking, abundant foam formation. This led to the suspicion that saponins were involved. In order to ascertain these various reactions of saponins were treid by using the SCHUMAN procedure (13). So as to determine the hemolytic value of seeds, and all of the reactions were positive. It is not a recent idea that saponins can regulate or influence germination (1-3, 9, 15). Besides, MARCHAIM (7), HARDMAN and WOOD (5) and SIGMUND (14), showed the influence of saponins on germination, with retardation or stimulation depending on their concentration.

The work reported in this article is an attempt to study the influence of cold stratification and germination on *Pinus*

pinea seeds with regard to their saponin level as well as the effects on the speed and percentage of germination of an excess of certain saponins introduced into the seeds without or with stratification.

Materials and Methods

Fifteen lots of *Pinus pinea* seeds from Coca (Segovia, Spain) were used. The soaking time for each seed lot in the liquids described below was 5 days.

Lot 1: Seeds soaked in distilled water. **Lot 2:** Seeds soaked in an aqueous solution with 1.17 g hecogenin acetate. **Lot 3:** Seeds soaked in an aqueous solution with 72.8 μg/ml of saponins extracted from *Pinus pinea* seeds. **Lot 4:** Seeds soaked in an aqueous solution of saponins extracted from *Pinus pinea* seeds at similar concentration, but diluted 1:10. The seeds for **lots 5, 6, 7** and **8** were prepared in a way similar to lots 1, 2, 3 and 4, but after soaking they were stratified for three weeks. **Lot 9:** Seeds soaked in an aqueous digitonin solution of 23.3 μg/ml. **Lot 10:** Seeds soaked in an aqueous digitonin solution of 23.3 μg/ml. **Lot 11:** Seeds soaked in an aqueous digitonin solution of 2.33 μg/ml. The seeds for lots **12, 13** and **14,** were prepared under the same conditions as lots, 9, 10 and 11, but they were taken out after five-day soaking and then strafied for three weeks. **Lot 15** was the control lot; so the seeds were neither soaked in any aqueous solution nor stratified afterwards.

The different stratifications of seeds took place at 4° C on vermiculite moistened with water.

Germination tests were carried out on non-stratified seeds (lot 15), on seeds taken out after soaking (lots 1, 2, 3, 4, 9, 10 and 11) and on seeds stratified after soaking (lots 5, 6, 7, 8, 12, 13 and 14) in a Jacobsen's chamber at 29° C with the lots containing 100 seeds each.

The stratification periods for the seeds subjected to saponin determination were as follows: 4 days; 1, 2 and 3 weeks; and 1, 1.5, 2.5 and 3 months.

For germinated seeds five stages were considered: freshly germinated seeds and seed whose seedlings have radicle lengths of 5-15 mm, 20-50 mm, 100-130 mm and 180 mm.

Saponin extraction. The external cover of the seeds was removed and the fat totally eliminated by means of a Soxhlet apparatus using ethylic ether. Finally the defatted seed residues were extracted with an 80 % ethanol solution up to the complete draining of the saponins.

Determination technique. Determination is based on making, with a spectrophotometer at 540 nm the measurement of absorption caused by hemoglobin set free by the hemolysis of erythrocytes (kindly supplied by the Spanish Centre for Hematology), washed together with the saponins contained in the material (4, 6). In order to check the calculation, measurements were taken under similar conditions of the absorption caused by hemoglobin set free through the effect of digitonin standards on red corpuscles.

Results

It was deduced that cold stratification generally causes an increase in the germination speed and percentage of the seeds even with seeds soaked in different saponin solutions. It was also shown that the saponin extracts from the seeds (lot 3) caused a decrease in speed and germination percentage as compared with the seeds only soaked in water (lot 1); but in diluted saponin solution (1:10) (lot 4), the decrease was lower; consequently, the dilution of saponin solutions influences germination. This means that an excess amount of saponins introduced into the seeds, partially hindered germination. Lot 2 treated with hecogenin acetate is a control lot and the degree to which germination

was hindered was visible from the 50th day after the seeds had been put to germinate (table I).

It was shown that until the 15th day of stratification the increase in the germination pe centage was practically the same in lots 5, 7 and 8; only the hecogenin acetate, in the lot 6 reduced the germination percentage. From the 15th to the 65th of stratification, some variations have been observed in lots 5, 7 and 8; the saponins coming from their own seeds partially hindered germination, inspite of being stratified, if compared with the seeds of lot 5 that were soaked in water and later stratified. The lot 8 showed that from the 50th day of stratification the diluted saponins extracted from the seeds stimulated the velocity of germination (table I).

Digitonin at various concentrations, showed little influence on the germination of non-stratified seeds. Besides the stratification of seeds soaked in digitonin solutions (lots 12 and 13) showed a significant germination increase when compared with the seeds from lots 9, 10 and 11 belonging to non-stratified, but was lower than in seeds soaked in water and stratified belonging to lot 5 (table I).

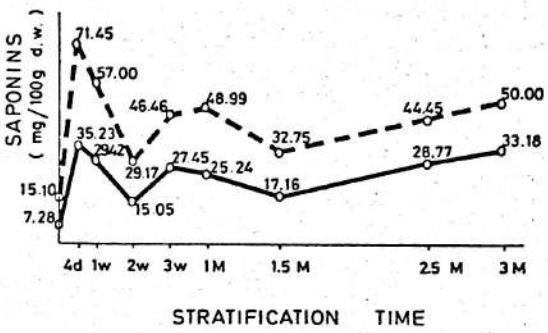

Fig. 1. *Curve of saponin contents of seeds stratified with distilled water.*
The ordinate represent saponins in mg/100 g dry weight. The abscissas, indicate the stratification time. — Saponins in dry weight. --- Saponins in defatted dry weight.

The results of saponin determinations in seeds, stratified and germinated are shown in figure 1 and figure 2. The saponin values refer to 100 g of dry defatted seeds apart from those refering to dry non-defatted seeds.

Table I. *Effect of various saponins at different concentrations on germination of non-stratified and stratified* Pinus pinea *seeds.*
Description of different lots in Materials and Methods.

Lot	Number of days in a Jacobsen's chamber at 29° C											
	1	10	15	20	30	40	50	60	65	70	80	90
	% of germinated seeds											
1	0	6	7	9	12	17	24	36	38	38	46	52
2	0	1	7	7	14	16	20	20	21	21	22	22
3	0	1	4	4	5	10	11	13	13	13	24	32
4	0	2	6	6	8	11	18	27	35	39	47	61
5	0	20	34	42	45	48	49	51	51	56	60	66
6	0	20	25	27	28	39	43	45	48	54	62	73
7	0	32	34	36	36	42	44	47	50	55	59	63
8	0	35	38	38	39	46	50	56	62	70	77	89
9	0	2	3	8	10	12	26	26	—	—	—	—
10	0	4	8	8	10	10	13	20	—	—	—	—
11	0	1	5	5	9	9	14	22	—	—	—	—
12	0	26	30	31	32	34	39	51	—	—	—	—
13	0	26	30	32	35	37	46	52	—	—	—	—
14	0	27	29	29	32	36	43	48	—	—	—	—
15	0	0	1	1	3	4	6	12	15	23	34	47

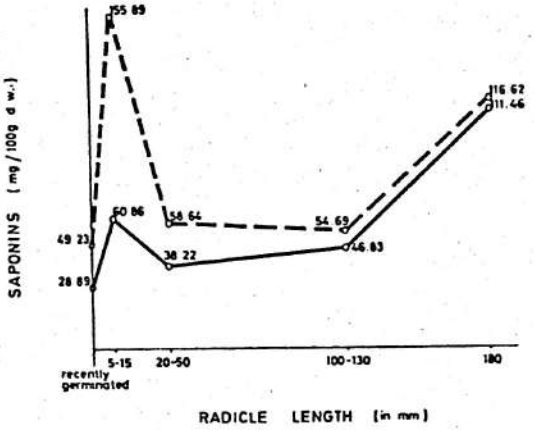

Fig. 2. *Curve of saponin contents in germinating seeds.*
The seeds were previously stratified for 15 days and later germinated in a Jacobsen's chamber at 29° C. The ordinate represent saponins in mg/100 g dry weight. The abscissas indicate radicle length of seedlings. — Saponins in dry weight. --- Saponnis in defatted dry weight.

As regards the saponins in figure 1, the stratification had an enormous influence on their amount and they reached their highest level around the 4th day of stratification; afterwards, the saponin contents decreased until the second week and increased again, gradually up to one month of stratification to decrease and then increase again.

Germination also caused an increase in the saponin level (fig. 2) which reached its highest amount during the second germination stage, i.e. up to the point where the radicle length of the seedlings reached 5-15 mm; then the contens decreased and remained at constant values for seedlings with a radicle length of 20-50 mm to 100-130 mm, only to increase again significantly.

Discussion

It is natural, that seeds soaked in a solution of hecogenin acetate (lot 2) should reduce the germination speed and percentage as compared with germination of seeds soaked in water, because hecogenin is a sapogenin foreing to the seed; the same holds true for seeds enriched by soaking in a solution of saponins extracted from the seeds themselves (lot 3); an excessive amount of saponins (lot 3) introduced into the seed slows dowm the germination, a fact which is confirmed both by lot 4 and the results reported by other authors, who point out that saponins at certain concentrations in some types of seeds retard or inhibit germination.

The previous three-week stratification to which lots 5, 6, 7 and 8 were subjected, showed a strong influence on the germination speed and percentage as compared with non-stratified lots 1, 2, 3, 4 and 15. Stratification (lot 5), not only favours germination in regard to dormant or non-stratified seeds (lot 15), breaking the dormancy, but also removes the germinative inhibition caused by the hecogenin acetate or an excessive amount of saponins (lots 9, 10 and 11). The facts stated above confirm once more that cold stratification is a conditioning, internally structural, seminal process which cancels out the action of inhibitors and helps the biochemical germinative mechanisms.

The fact that saponins are present in seeds that are edible is due to the smaller amount of these substances (7.28 mg/100 g = 72.8 μg/g) which make them tolerable.

Furthermore, it could seem that the saponin quantitative variations occurring during the stratification and germination processes show that saponins are involved in the seminal metabolism, and consequently they are not a ballast material.

Resumen

Se determinan saponinas en semillas de *Pinus pinea* no estratificadas, estratificadas sobre vermiculita humedecida a 4° C, en semillas puestas a germinar en cámara de germinación Jacobsen a 29° C y en plántulas con radículas de longitudes comprendidas entre 1-130 mm. La estratificación produjo un elevado aumento de

las saponinas con un máximo nivel al cuarto día de comenzar y otro a las tres semanas de estratificación manteniéndose más bajos pero mayores que los del comienzo de la estratificación.

La germinación produjo dos incrementos significativos de 60,86 mg/100 g de peso seco, al alcanzar la radícula de la plántula una longitud de 5-15 mm y después a partir de plántulas de radícula de 100-130 mm.

Un exceso de saponinas introducidas en semillas de *Pinus pinea*, por inmersión de éstas en las soluciones correspondientes a determinadas concentraciones origina una disminución en el porcentaje de germinación, pero cuando el exceso introducido es a concentraciones menores que aquéllas lo estimula.

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