An. Real Acad. Farm. 1979, 45: 581-588

Chlorophylls in Pinus pinea Germinating Seeds

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Summary

Chlorophylls *a* and *b* were identified and determined: in embryos of *Pinus pinea* seeds, stratified for 60 days at 4 °C on vermiculite moistened with water; in seeds stratified put to germinate into a Jacobsen's chamber, for 2; 3 or more days and in seedlings from seeds germinated. As standards, chlorophylls *a* and *b* extracted from spinachs were used. For separation of chlorophylls, sucrose columns and thin layer chromatography were used. Identification was made by coloured bands of columns, by the Rf of chromatogram spots and absorption maxima. Determinations were made by spectral measurement of extracts. It was concluded that germination increases the level of chlorophylls *a* and *b* in a gradual and significant way with respect to pre-germinated seeds.

Introduction

In our Department qualitative and quantitative changes of different constituents of *Pinus pinea* seed were studied through cold stratification and germination (1, 5, 6, 7, 8, 9, 10). In coniferous plants during germination chlorophyll ussually is biosynthesised, in *Pinus pinea* seed chlorophyll is biosynthesised too. For this reason it was interesting to study the stage in which the biosynthesis of chlorophyll or chlorophylls began to follow the quantitative course of those compounds in the earlier stages of the development of seedlings, and also to identify what chlorophylls there are in the seed.

Identification of chlorophylls is usually made by means its absorption spectra after extraction of the samples with certain solvents and farther separation of chlorophylls by column adsorption or thin-layer-chromatography. In this work, the chlorophylls extracted from fresh spinach were used as standards in comparison with those extracted from embryos or seeddings of *Pinus pinea* seed.

Material and methods

The material used in the tests was *Pinus pinea* seeds from Coca (Segovia, Spain), with a germination capacity of 95 %. The stratification was made at 4 °C on vermiculite moistened with water at different dates or periods. Vermiculite and seeds were arranged in alternating layers. The germination tests were carried out in Jacobsen's chamber at 28 °C with seeds stratified for 15 days at different stages of germination by taking the following radicle lengths as models: 0-5; 5-10; 10-15; 20-30 mm and longer than 30 mm.

Preparation of pigment extracts from fresh spinach:

For column chromatography:

0.7-0.8 g. of fresh spinach were triturated with help of washed sand in presence of acetone or other solvent as ethanol or methanol and centrifuged at 6000 r/m (r. p. m.). The residue was extracted and centrifuged again until total exhaustion was obtained. The supernatan solutions were decanted and evaporated in current of nitrogen and the total residue dissolved in petrol-ether (B. P. 30-40 ° C), was used for separation and identification of chlorophylls by means of column chromatography.

For thin-layer chromatography:

The extracts were prepared of similar way as for those obtained for column chromatography, using methanol as solvent until total exhaustion. The methanol solution was divided in three parts and the solvent of two removed in current of nitrogen. The residues were dried for 24 hours in presence of Cl_2Ca and dissolved respectivaly in petrol-ether and ether for application on the starting point. Thus there are three solutions prepared for the making of chromatograms in thin-layer.

Preparation of pigment extracts from embryos or seedlings of *Pinus pinea* seeds:

For column chromatography and thin-layer-chromatography:

The extracts for testing were prepared in a similar way as in those extracts obtained from fresh spinach; but was used methanol as solvent. In order to avoid the extraction of lipids (*Pinus pinea* seed contains about 51 %), methanol was used as solvent. The solvent was removed in current of nitrogen and dissolved in petrol-ether (B. P. 30-40 °C). Each solution was divided into two parts; the former was applied for column chromatography and the latter for thin-layer-chromatography.

Identification of chlorophylls

In extracts of spinach

Separation of chlorophylls by means of column chromatography

As materials were used, adsorbent, sucrose: starch (97: 3) finely ground and activated for 12 hours at 85 °C; solvent for development petrol-ether (B. P. 30-40 °C): n-propanol (99,5: 0,5).

The bands obtained were as follows: 1, Yellow, 2, Bluish green; the colour, location and absorption maxima of this pigment proved to be chlorophyll *a*. 3, Yellow. 4, Yellowish green. The colour, location and absorption maxima of this pigment showed to be chlorophyll *b*. 5, Yellow. 6, Yellow. The position of the yellow spot, number 6 is on the top of the column. A lot of chromatograms were prepared from methanolic and acetonic extracts from fresh spinach and were all similar.

The bands 1 and 2 of the column were removed by elution with petrol-ether (B. P. 30-40 C containing 0.5 per cent of n-propanol). The pigments 5 and 6 were removed through the opening of the column with help of a small rod and the pigment 4 was eluted with petrol-ether containing 3 per cent of ethanol. The solvents of eluates 2 and 4 were evaporated in current of nitrogen until dryness. The residues of the solutions in methanol were taken for the measurement of their absorption spectra.

In extracts from embryos and seedlings of *Pinus pinea* seeds, separation and identification of chorophylls were made of similar way to that of spinach verified by means of column chromatography and thin layer chromatography. The bands obtained were: 1, Bluish green, the colour, location and absorption maxima of this pigment proved to be chlorophyll *b* 3, Yellow.

Separation of chlorophylls by means of thin layer of chromatography

This is based in the Bunt's technique (1964) for estimating of chlorophylls in algas and used in this work for spinach and seedlings. See Fig 1.

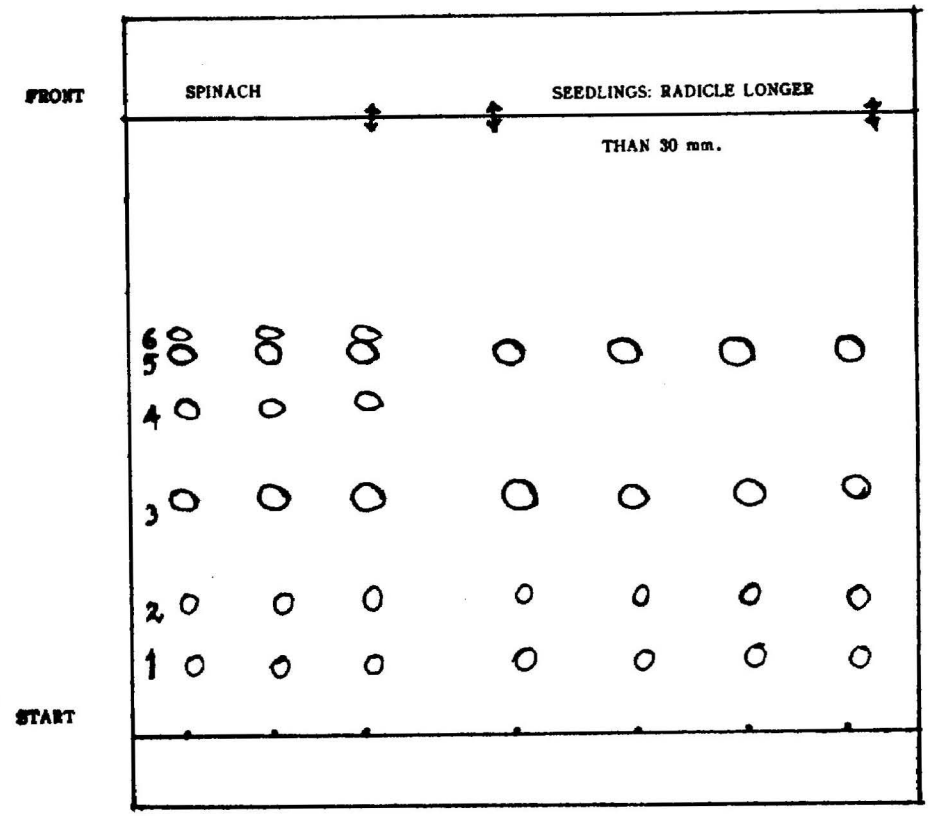

Fig. 1.—Average chromatogram representative of many carried out with the pigments that came from:

Methanolic extracts from fresh spinach used as standards:

Spots: 1 Yellow. 2 Yellow. 3 Yellowish green. The colour, location and absorption maxima of this pigment proved to be chlorophyll *b*. 4 Yellow. 5 Bluish green. The colour, location and absorption maxima of this pigment showed to be chlorophyll *a*. 6 Yellow.

Methanolic extracts from seedlings of *Pinus pinea* seeds with radicle longer than 30 mm:

Spots: 1 Yellow. 2 Yellow. 3 Yellowish green. The colour, location and absorption maxima proved to be chlorophyll *b*. 5 Bluish green. The colour, location and absorption maxima showed to be chlorophyll *a*.

Average Rf: values of principal spots: 6 = 0.98. 5 = 0.96. 3 = 0.87.

Adsorbent: Kieselguhr G (Merck-Stahl). Plates 20 × 20 cm. Layer thickness 0.5 mm.

Activation of layers at 110 °C for 1 hour. Solvent-system for development of chromatograms, petrol-ether (B. P. 65-90 °C): n propanol (99:1).

The spots 5 (bluish-green) and 3 (yellowish-green) obtained by thin layer of chromatography (see Fig 1) from spinach and seedlings of *Pinus pinea* seeds have similar Rf. The spot colours, Rf values and absorption maxima 435, 665 and 470 and 650 nm showed to be chlorophylls too.

Determination of chlorophylls

They were accurately weighed in each determination of 0.5-1 g of fresh spinach on *Pinus pinea* seed embryos, and extracted with methanol in a similar way to that for the identification of pigments.

The residues were dissolved in 5 ml of 90 % acetone and its absorptions measured at 649 and 665 nm (Gottschalk and Müller 1964, Vernon 1960 y Mc. Kinney 1941).

Chlorophyll *a* was determined by the equation:

Chl *a* (μg/ml) = 11.63 (A_{665}) — 2.39 (A_{649}).

Chlorophyll *b* was determined by the equation:

Chl *b* (μg/ml) = 20.11 (A_{649}) — 5.18 (A_{665}).

The sum of values obtained for chlorophylls *a* and *b* must be similar to that obtained applieing the equation:

Whole chlorophyll μg/ml = 6.45 (A_{665}) + 17.72 (A_{649}).

A represents the optic densities at 665 nm and 649 nm and the values indicates μg per ml.

The quantitative results of chlorophylls *a* and *b* in relation to development of seedlings are described in the Table 2.

Results

In order to identify the chlorophylls of *Pinus pinea* seeds by their absorption spectra a large quantity of seed extractions were carried out. The solvent used was methanol. The extractions and spectral measurements were made in: *Pinus pinea* seed subjected to cold stratification for 60 days; in seed put for germination in Jacobsen's chamber (after stratification for 15 days) 2; 3; 22 and 24 days and in seedlings with radicles length of 0-5; 5-10; 10-15; 15-20; 20-30 mm. In some cases the extracts were first run through a sucrose column. See the Table 1.

TABLE 1

Identification of chlorophylls

Pinus pinea seeds	Absorption maxima (Peaks) (*)	Solvent for extraction and measurement	Observations
Stratification for	In Embryos		
60 days	430; 436; 470; 665 nm	Methanol	The peak in 665 is little perceptible in the three determinations.
60 days	415; 436; 470; 665 nm	Methanol	
60 days	420; 435; 470; 665 nm	Methanol	
Put for germination (After stratification for 15 days)		In seedlings	
2 and 3 days	440, 470 nm	Methanol	The peak in 665 is little perceptible in all determinations.
22 days	440; 470; 665 nm	Methanol	
24 days	440; 470; 665 nm	Methanol	
Radicle length:			
0- 5 mm	435; 470; 615; 665 nm	Methanol	
0- 5 mm	435; 470; 615, 665 nm	Methanol	
5-10 mm	430-435; 436; 470; 620; 665 nm	Methanol	
5-10 mm	415; 435; 625; 665 nm	Methanol	
5-10 mm	410; 470; 660 nm	Methanol	
10-15 mm	415-420; 435-440; 470; 615; 665 nm	Methanol	
15-20 mm	435-440; 470; 615; 665 nm	Methanol	
20-30 mm	435; 470, 615; 665 nm	Methanol	

(*) The peaks underlined are correct bearing in mind that the absorption maxime of chlorophyll *a* is the range 432-435 and 665 nm and the peaks of chlorophyll *b* in 470 and 650 nm. The peak in 415 nm is a slight inflection of the chlorophyll *a*.

TABLE 2

Chlorophyll per g/referred to dry weigh

Seeds stratified for 15 days and:	Chlorophylls a	b	a + b	Total chlorophyll
	In embryos			
Put in Jacobsen's chamber for 2 days	1.45 µg	2.35 µg	3.80 µg	3.80 µg
Put in Jacobsen's chamber for 3 days	2.69 µg	9.73 µg	12.43 µg	10.80 µg
	In seedlings			
Germinated in Jacobsen's chamber:				
Radicle length 0- 5 mm	0.14 mg	0.06 mg	0.20 mg	0.21 mg
Radicle length 5-10 mm	0.34 mg	0.16 mg	0.50 mg	0.50 mg
Radicle length 10-15 mm	0.37 mg	0.12 mg	0.49 mg	0.50 mg
Radicle length 15-20 mm	0.39 mg	0.11 mg	0.50 mg	0.50 mg
Radicle length 20-30 mm	0.71 mg	0.32 mg	1.03 mg	1.03 mg
Radicle length longer than 30 mm	1.02 mg	0.43 mg	1.45 mg	1.46 mg

DISCUSSION

Chlorophylls from embryos or seedlings of *Pinus pinea* seed, obsviously are *a* and *b*; because not only have they been identified by their absorption spectra but also they have similar Rf with regard to chlorophylls from spinach taken as standards and the suitable bluish-green and yellowish-green colours of the spots in chromatograms obtained by thin layer-chromatography (Fig 1).

On the other hand, the separation of chlorophylls from seedlings through sucrose column, produce two bands of bluish-green (1) and yellowish-green (2) colours, similar to bands (2 and 4) that come from the spinach. Separation of chlorophylls *a* and *b* from seedlings is not so sharp or clear as in the chromatograms obtained of spinach extracts; probably because the yellow pigment (3) increases the migration of the chlorophyll *a* whereas the seedlings does not show this one. However the absorption maxima of chlorophylls separated

by this procedure from seedlings, coincide almost exactly with those that came from the spinach.

Methanol was used as solvent for extraction of chlorophylls from embryos and seedlings because it practically does not dissolves the lipids contained in them; in addition it is as good a solvent of chlorophylls as acetone or ethanol.

In seeds dormant (non-stratified) and stratified, chlorophylls did not appear; but later their biosynthesis was initiated in pre-germination before the processes of growth the could lead to protrusion of part of the embryo through the seed cosering. The increase of the chlorophylls is naturally later than the germination progress and development of the seedlings.

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KEY WORDS: *Pinus pinea*. Seeds. Chlorophylls. Thin-layer. Chromatography (TLC) Germination. Cold stratification.

An. Real. Acad. Farm., 1979, 45: 589-598

Sobre la flora y corología de la Serra da Estrela (Portugal)

por

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RESUMEN

Se delimita el sector corológico Estrellense en base a sus peculiaridades geográficas, geomorfológicas, climáticas, edáficas, florísticas y fitosociológicas. Asimismo, se hace un análisis del elemento y subelemento endémico, sectorial, provincial y de mayor área, así como se sugieren las relaciones florísticas y migratorias con otros territorios peninsulares. Por último, se dan a conocer algunas novedades florísticas para la Serra da Estrela, de las que algunas lo son también para Portugal.

SUMMARY

The limits of the Estrellense chorologic sector are defined by means of its geographic, geomorphologic, climatic, edaphic, floristic and phytosociologic features. An analysis of the endemic element and subelement at the sectorial, provincial and wider levels is performed, and the floristic and migratory relationships with other peninsular territories are suggested. Lastly, some floristic novitates for Serra da Estrela are presented, some of which are also new for Portugal.

Hace cinco años uno de nosotros publicó un pequeño artículo «Datos sobre la flora y la vegetación de la Serra da Estrela (Portugal) - Anales de la Real Academia de Farmacia, 40 (1): 65-74», en el que trataba de dar a conocer algunos datos fitosociológicos y florísticos de dicho macizo con ánimo de destacar sus relaciones con las altas montañas de la Cordillera Central y con las de otros territorios de la provincia corológica Carpetano-Ibérico-Leonesa.

A pesar de que un lustro no es un plazo de tiempo demasiado largo, algunas campañas botánicas realizadas desde entonces a tra-