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DEVELOPMENT OF QUICK METHODS FOR PRODUCING OF SCLEROTIA AND APOTHECIA OF SCLERO - TINIA SCLEROTIORUM IN LABORATORY

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SUMMARY

Quick methods for producing sclerotia and apothecia of Sclerotinia sclerotiorum in laboratory are described. For fast and large amount of sclerotia production, and among the natural substrates assayed, carrot substrate proved to be the most suitable, producing large sclerotia (1.5-2 cm) in 28 days. About 100 percent carpogenic germination of sclerotia was obtained by planting them in soil kept moist or subjecting them to low temperatures during 3 days followed by incubation under high moisture conditions and absence of light. Apothecial stipes were formed in 38 days (soil) or 23 days (pretreatment of low temperatures). Apothecia development under high moisture and laboratory conditions of light and temperature, was attained in 5-7 days, presenting discs up to 8 mm in diameter (average dimensions 5-6 mm). According to the methods studied, apothecia of first generation could be obtained in one month, being of about two months the total elapsed time to produce sclerotia and apothecia in laboratory. Viability of the ascospores released "in vitro" was verified.

INTRODUCTION

Among sunflower diseases reported in Portugal (Barros 1980; 1986) particular emphasis is given to Sclerotinia sclerotiorum due to the importance of this fungus towards sunflower and other crops of economic importance. In order to develop studies on its epidemiology and aiming at the selection of cultivars resistant to this fungus, it is of utmost importance to handle a great amount of apothecia wich is not always easy to observe in nature.

So, based on the relevant literature on the subject, the authors have selected from the techniques described some aspects, having tried to associate and improve them, aiming at a faster production of sclerotia and apothecia in laboratory. Some important aspects such as the regularity in the production of the above referred structures, their number independently from the season as well as the ulterior production of ascospores and their viability have been considered with particular care. Together with the development of the techniques for the production, of the different structures of the S. sclerotiorum life cycle, the authors assume they are also contributing to a better insight of the biology of the fungus and of the factors involved in the carpogenic germination.

MATERIAL AND METHODS

Biological material

The authors have based their studies on pure cultures of S. sclerotiorum grown in PDA after being isolated in July 1983 from infected stems of Helianthus annuus, collected in the field trials of the Instituto Superior de Agronomia (ISA),Lisbon.

Methods

Production of sclerotia "in vitro"

As we needed a large amount of sclerotia it was not practical to grow the fungus in petri dishes or tubes and so, according to Lamarque (1980), the performance of three substrates (potato, carrot —Keay, 1939— and onion) without adjustment of their natural pH was compared. A similar technique was used to prepare the substrates. Sterilized pieces (cut into cubes) of potato, carrot and onion (100g of each/ Erlenmeyer or Roux flask, at 0.5 atm. during 20 min. being the operation repeated for 3 times at 24 hours intervals) were incubated at $18^{O} \pm 1^{O}C$ after being inocu-

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lated with one sclerotium of S. sclerotiorum. As more than one generation of sclerotia is formed the period of incubation was considered as finished when an amount of recently formed sclerotia already mature (pigmented) was floating on the liquefied substrate, though some diminutive traces of the solid substrate and very few mycelia could be observed, but only if sclerotia primordia were not conspicuous. Sclerotia were separated by using a sieve under tap water. The "stocks" of sclerotia, dried naturally were introduced into perforated plastic flasks, under the laboratory natural conditions and stored for further use without any particular asepsis care. Sclerotia obtained by the above conditions were subsequently submitted to carpogenic germination'trials.

Carpogenic germination

There are several confusing reports on the minimum resting period needed by sclerotia of Sclerotinia spp. before carpogenic germination can take place. This resting period is of apparent inactivity and referred to as dormancy (Coley-Smith & Cooke, 1971; Willetts & Wong, 1980).

We have based our investigation on a deep review of literature on factors affecting apothecial production such as light, temperature, age of sclerotia, nutrient status of substrate and moisture. The substrates utilised for this purpose were very diversified (from distilled water to water agar and even poor or richer agar media containing vegetable extracts or synthetic ones, and moist substrates like sand, soil, mixture of soil with sand and other synthetic substrates as wet cotton or perlite). After having recognized that the essential feature was the maintenance of a more or less constant moisture supply during a certain period of time, we have developed the following methodologies:

– In soil (open air condition)

Sclerotia of the "sotck" obtained in the laboratory were placed in small plastic sieves and buried into unsterilized ordinary garden loam, 2 cm deep, in ordinary flower pots with good drainage, placed in the open air. Soil moisture was kept high and more or less constant by watering the pots with tap water, on the absence of rain. Once apothecial stipes formed, parent sclerotia were carefully extracted from the soil and separated under running tap water.

– Pretreatment of low temperature

Based on Ale-Agha (1974), stored sclerotia formed in the 3 natural substrates were introduced into plastic bags and submitted to temperatures of - $5^{O}\pm 1^{O}C$ in an ice refrigerator during 1, 2 or 3 consecutive days, in the absence of light to remove dormancy. Pretreated sclerotia were then put into petri dishes with wet cotton covered by filter - paper at $18^{O} \pm 1^{O}C$ in the absence of light.

Development of apothecia

If sclerotia with apothecial stipes are held in darkness, the stipes go on growing and their apices develop each a single apothecium only if exposed to light (Henderson, 1962). There are other features such as temperature (15^{O}-$20^{O}C$), Coley-Smith & Cooke (1971) and a constant high moisture as for production of the stipes.

Two techniques were assayed. The first one, according to Henson & Valleau (1940) technique (modified) consisted in pressing parent sclerotia with well formed stipes ($\pm$ 0.5 cm long) into sterile 1% and 0.5% water agar slanted in test tubes of $\pm$ 2 cm of diameter kept in the natural conditions of the laboratory. With regard to the second one, a similar lot of parent sclerotia with stipes was placed into covered crystallizing dishes ($\pm$ 9 cm diameter x 5 cm high) and / or pyrex weighing bottles with emery polished lids (7 cm diameter x 6 cm high) with wet cotton covered by filter-paper. Half of them were placed under any desired condition of temperature and light in a growth cabinet (we used 15^{O} C and 5 000 lux) in alternate day and night, and the remaining were put in laboratory natural conditions of temperature and light. In another experiment the second technique was performed under laboratory conditions of light and temperature with two lots of sclerotia bearing stipes ($\pm$ 0.5 cm). One of the lots was deprived from the stipes to enable to evaluate the effect of cutting off the first generation stipes on the second generation stipe formation (Lamarque, 1980).

Ascospores

In considering viability of ascospores also two techniques were compared.

The first one (Lamarque, 1980) consisted in inverting mature apothecial discs over sterilized confetti like filter-paper discs inside petri dishes. The ascospores were discharged on to the paper discs and these aseptically introduced into PDA slanted test tubes. The second technique consisted in aseptically trapping during few seconds the ascospores in PDA plus PST petri dishes inverted on the glass containers with sclerotia bearing mature apothecia. Both test tubes and petri dishes were incubated at $25^{o} \pm 1^{o}C$.

RESULTS AND DISCUSSION

For the production in laboratory of sclerotia and apothecia of S. sclerotiorum we considered of utmost importance the facility, rapidity, efficacy and reproductibility of the methods. Efficacy and rapidity in production of sclerotia can be verified in table I.

TABLE I - Production of sclerotia "in vitro"

Substrate (wet weight) (100g)	Period of incubation (days)	Max. dimensions of sclerotia (cm)	Average weight of sclerotia produced (dry weight) (g)
Potato	52	1.0 x 0.5	22.5
Carrot	28	2.0 x 1.0	18.2
Onion	43	0.5 x 0.4	6.7

From table I it is easily verified that better efficacy in sclerotia production was attained with potato immediately followed by carrot substrate. Notwithstanding carrot substrate proved to be more suitable, producing larger sclerotia (1.5-2 cm) in less days (Plate I, fig. 1). So, we saved some time when compared to Lamarque's results (1980) in this phase of the life cycle of S. sclerotiorum. As this fungus in the field usually forms apothecia in determined environmental conditions, we can infer that apothecial production comes under environmental controls or, that removal of dormancy is brough about by special stimuli. So, at least some of the resting period was taken up with the development of apothecial initials within parent sclerotia being those initials formed just prior to emergence or alternatively, some time earlier, but then pass through a period of quiescence until exogenous factors or conditions become favourable for renewed growth (Willetts & Wong, 1980). As we intended to accelerate the carpogenic germination also, we have carried out our assays with sclerotia formed "in vitro" immediately after they have been produced. This fact did not affect in anyway the further production of apothecia.

In the experiments done in soil conditions we have kept in mind some practical aspects, by employing unsterilized soil (Lamarque, 1980) and using plastic sieves (Pierre & Regnault, 1985) which facilitates the ulterior removal of sclerotia, diminishing in fact the damages towards the recently formed apothecial stipes. About 100 percent carpogenic germination of sclerotia was obtained by planting them in soil kept moist and we have observed apothecial stipes well formed in 46, 38 and 50 days in parent sclerotia produced respectively in potato, carrot and onion substrates. As referred by Coley-Smith & Cooke (1971) there is a relation between sclerotium volume (and weight) and the number of apothecia formed, so it is not surprising that we produced stipes faster and in larger amounts, in parent sclerotia formed in carrot, which were the largest and more vigorous ones. Concerning the period of burial in the soil there was an economy of time in relation to Lamarque's (1980).

The pretreatment with low temperatures under darkness to remove dormancy has shown the efficacy of any of these periods but only 30-40 percent of sclerotia formed stipes afterwards, in the 2 first cases. However 3 days of low temperatures induced the ulterior production of apothecial stipes in 100 percent of

parent sclerotia after more or less 20 days of incubation at 18° ± 1°C and high moisture conditions. Comparing these two techniques the second is quicker, but the stipes produced in the soil were in higher number and more vigorous.
Concerning the disc formation (mature apothecia) "in vitro" the authors corrobor ated some previous observations like that sclerotia in presence of rich substrates formed mycelia while poor substrates favoured carpogenic germination (Ale-Agha, 1974). In what concerns the two techniques assayed the use of weighing bottles with wet cotton covered with filter-paper was the best. In fact this sub strate, permitting a constant high moisture is not injurious to the stipes, it does not prevent their development towards the light source and avoids the alteration on sclerotia relative positions, when manipulated, which does not happen in the crystallizing dishes containing only water.
Regarding the vials with nutrient medium we have preferred sterile 0.5 percent water agar due to its consistance, but this technique, considering our purposes is less efficient both in what concerns the number of sclerotia produced per tube and the time spent with their manipulation. Previous results for disc formation under high moisture and natural laboratory conditions of light and temperature were similar to those obtained in the growth cabinet. Apothecia presented buff to rosy buff (Rayner, 1970) discs up to 0.8 cm, the average diameter of the discs from parent sclerotia formed in carrot, potato and onion being respectively 0.5 - 0.6 cm in the first case and 0.3 - 0.4 cm in the two last ones.
Table II summarizes the total elapsed time to produce mature apothecia from parent sclerotia formed in carrot substrate. The sclerotia bearing apothecial stipes of first generation were put into weighing bottles under high moisture and natural laboratory conditions of light and temperature.

TABLE II - Production of apothecia (1st generation)

Techniques to remove sclerotia dormancy	Number of days		
	Production and development of apothecial stipes	Formation of apothecial discs (mature apothecia)	Total
In soil	38	5	43
pretreatment of low temperatures	23 (3 + 20)	7	30

If we add to the above periods the number of days necessary for the formation of sclerotia "in vitro", slightly below one month (in the case of carrot substrate), it is clearly demonstrated that approximately 2 months was the total elapsed time to produce sclerotia and perfect as well as vigorous apothecia (Plate I, fig. 2 to 4) in laboratory. According to the available literature it is apparent that the process described is faster than those referred by other research workers.
The elapsed time between the two first generations of apothecia is about 11 days and all the techniques assayed to produce apothecia have been accompanied by viability tests of the ascospores released "in vitro", being more practical the second technique developed by the authors.
The ascospores produced mature sclerotia after 10 days of incubation.
Great part of the literature consulted does not refer to ascospore viability tests.
We consider this as an essential point in order that any technique developed for the "in vitro" production of apothecia could be applied in further studies of epidemiology and varietal selection concerning resistance to S. sclerotiorum.

GENERAL CONCLUSIONS

Since our final objective was the development of quick methods for production of sclerotia, apothecia and viable ascospores of S. sclerotiorum, the compared

methodologies carried out relatively to the different structures of the fungus life cycle have brought out the following pertinent conclusions:

– Among the natural substrates assayed, carrot substrate proved to be the most suitable. Although producing an amount of sclerotia slightly lower than that formed in potato, the sclerotia were of higher dimensions (1.5-2 cm) and were produced in a shorter period (28 versus 52 days). The poorest production was observed in onion substrate.

– Carpogenic germination has been observed in sclerotia buried in soil or submitted to pretreatment of low temperatures, immediately after having been formed "in vitro". Apothecial stipes (soil) or apothecial initials (pretreatment with low temperatures) were formed in dark conditions, but their expansion to form apothecial discs requires a light source.

– The technique of low temperatures proved to be the quickest (23 days) but apothecial stipes produced in the soil (38 days) were higher in number and more vigorous.

– Apothecial discs were expanded in 5-7 days under high moisture and laboratory natural conditions of light and temperature, attaining diameters up to 0.8 cm (average dimensions 0.5 - 0.6 cm).

– According to the general investigation performed, mature apothecia of first generation could be obtained "in vitro" in 1 month. About 2 months was the total elapsed time to produce sclerotia and apothecia in laboratory, the elapsed time between the two first apothecial generations being of about 11 days.

– Time is saved if the assays are performed with the first generation stipes. Better results were obtained if instead of cutting them off, they were not left to etiolate.

– Whenever apothecia were formed the viability of ascospores was checked and this is a requirement of the utmost importance for further investigations on epidemiology or selection of resistant cultivars.

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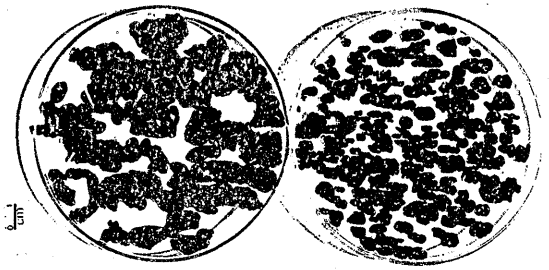

Fig. 1

Fig. 2

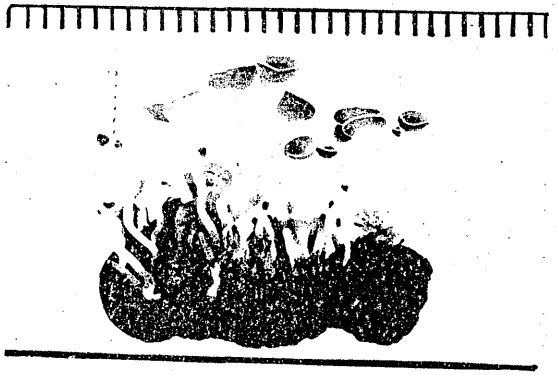

Fig. 3

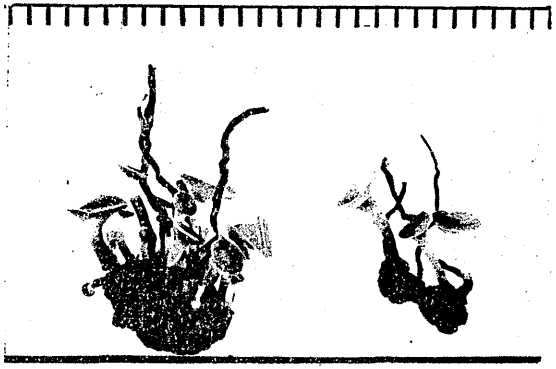

Fig. 4

PLATE I

(by M.M. Ferreira-Pinto & Maud L. de Barros)

The photos of our S. sclerotiorum material were taken by the Agronomists M.R. Proença-Santos (fig. 1) and Gonçalves Passos (fig. 2, 3 and 4)