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PERFECTING TEST METHODS FOR SENSITIVITY TO *PHYTOPHTHORA* FOR USE ON CACAO SEEDLINGS AND ON OTHER DICOTYLEDONOUS PLANTS WITH EPIGEOUS DEVELOPMENT

G. BLAHA and R.A. MULLER
Laboratory of Phytopathology
I.F.C.C., B.P. 5035
34032 Montpellier Cedex, France

(I) SUMMARY

In order to assess the respective aggressivity of various geographical isolates of Cacao *Phytophthora*, sensitivity tests are made on very young Amelonado plants and also on a range of plants originating from either temperate or tropical countries.

The successive trials on very young Cacao seedlings described here show differences in pathogenesis between isolates and open the way to a breeding technique in the laboratory and lead to improvement of the infection method through its standardization.

If a whole range of different plants is subjected to infection, it is possible to characterize several strains in relation to the same plant and also a single strain in relation to several plants.

The establishment of a range of differential hosts and knowledge of pathogenesis in Cacao would, in particular, make it possible to determine the level of parasitic specialisation for each isolate.

(II) INTRODUCTION

The IFCC Phytopathology laboratory at Montpellier has made a world collection of strains of cacao tree *Phytophthora* with the aim of contributing to a clarification of the differences between these organisms taking as a basis criteria other than morphological ones. The criteria used in this differentiation are cytological, physiological and pathogenic.

Here we are only concerned with these from the double standpoint of aggressiveness vis-à-vis cacao, on organs other than pods, and of parasite specialization on a range of differential hosts.

Evaluation of aggressiveness vis-à-vis cacao of these various geographically distinct isolates and of their parasitic specialization has necessitated the development of infection tests, on the one hand on very young Amelonado seedlings and on the other, on a range of plants originating either in temperate or in tropical countries.

(III) STUDY OF *PHYTOPHTHORA* AGGRESSIVENESS TOWARDS CACAO : INFECTION OF ROOTS OF YOUNG SEEDLINGS

The adoption of a method of infection rests on the choice of the type and means of inoculum application.

Several infecting methods have been tried out on very young seedlings. Although based on the method used in Ghana (1), (2), (3) and (4), and in Ivory Coast (8) on pre-germinated beans, they are essentially different in that the cotyledons are kept from any contact with the inoculum and that the root system, which alone is subjected to attack, is already at a well differentiated stage.

To discover the pathogenic strength of a strain the clearest and most easily observed evaluation criterion is the mortality rate : it is this criterion which appears most frequently in this paper. The vegetative state of survivors also enables us to evaluate this aggressiveness : for example, a slowing down of growth apparent in reduction of the plant's overall height, shortening of the internodes, reduction in the number of leaves and, in general, a drop in the dry weight of the aerial parts as compared with the non-infected controls.

1 - Plant material

The cacao trees used are "Amelonados" from Bingerville (Ivory Coast) which can be considered relatively homogeneous genetically speaking, and whose sensitivity to the local *Phytophthora* has been recognized from artificial infection tests on the pods in the plantation.

The beans are put to germinate in sterile vermiculite and kept damp with sterile distilled water. Germination takes place at an ambient temperature varying between 25 and 30°C. Infection is carried out during emergence, that is, 8 to 10 days after germination. At this stage of development, the well differentiated root system shows a taproot and a network of fine lateral rootlets.

2 - Type of Inoculum and Mode of Application

Three strains of *Phytophthora* , highly distinctive in their geographical origins and morphological character will enable us to illustrate the influence of the type of inoculum : one strain from Brazil (B_1), one from Ghana (G_7) and one from Cameroon (YN).

The pathogenic strength of a zoospore suspension of these three strains was compared with that of the mycelium paste.

a) Infection by zoospores

The zoospore suspension is prepared from a culture on gelosed V_8 at 1.5% and calibrated at 300-400 z/ml. The root system is either completely immersed in this solution for about one minute, or inserted in moistened vermiculite into which the zoospore suspension has been incorporated. In either case the seedling is planted out in a plastic pot with the base standing in distilled water. After eleven days, the highest mortality rates are given by G_7 and B_1, the lowest by YN.

Table 1 here

Graphs 1 and 2 here

b) Infection by crushed mycelium culture

The *Phytophthora* strains are introduced on to liquid V_8 at the rate of 80 ml per flask, and kept in the dark for 20 days at a temperature of 25°C. These culture conditions have the advantage of producing an almost exclusively vegetative mycelium ; development in a liquid medium and in the dark greatly reduces the formation of sporocysts, and shaking the cultures every other day prevents the liberation of zoospores during grinding. On the day of infection the cultures are washed in sterile distilled water and wrung out on to fritted glass filters. The retained mycelium is weighed after maximum resaturation, then ground for a minute in the proportions of 10 g to 100 ml sterile distilled water. The paste is diluted 5 times when infecting is carried out.

The culture may be applied in two ways : either by pouring 10 ml of suspension through a pipette directly on to the fine surface roots, or indirectly by mixing it into the vermiculite in which the young seedling is transplanted : 120 m of mixture and 300 ml of dry sterile vermiculite, carefully mixed together, are sufficient to fill a plastic pot 9 x 9 cm across and 10 cm deep in which 4 Amelonado seedlings will be planted. 5 pots (or 20 seedlings) per strain are prepared in this way for testing.

With the first type of infection the inoculum is more or less concentrated at the base of the hypocotyl and part of the root system may not be attacked, notably the tap root. One can say that with this type of infection, attack is localized.

With the second method, infection is less localized, all the roots being in contact with the inoculum to an equal degree. One can say that with this type of infection, attack is generalized.

3 - Results and Conclusions

Eleven days after infection the mortality rates reveal a more clearcut distinction in the aggressiveness of the strains when the mycelium mixture is applied using the second technique.

Table 2 here

Graphs 3 and 4 here

- The results obtained with the different types of infection show the strains in the same order as regards the virulence of their attack on cacao tree roots :

G_7 B_1 YN

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- However, distinction between strains G_7 and B_1 when infection is carried out with zoospores is difficult : these two strains seem to show a very similar high degree of virulence compared with YN.

- Likewise, B_1 and YN seem to have a very similar low degree of virulence when the mycelium culture is localized at the base of the hypocotyl.

- On the other hand, a clearer differentiation of the strains is apparent when the entire root system is subjected to attack by a mycelium mixture uniformly distributed through the substratum. It should also be noted that it is still possible to re-isolate the fungus from the mixture and vermiculite mixture even a month after the experiment has been conducted.

We conclude from this that the method of infecting the root system by a mycelium mixture incorporated into the substratum seems to be the most satisfactory for carrying out systematic aggressiveness tests. This method is also more readily standardized.

(IV) STUDY OF PARASITIC SPECIALIZATION IN *PHYTOPHTHORA* : INFECTION ON DIFFERENT DICOTYLEDONOUS PLANTS WITH EPIGEOUS DEVELOPMENT

The degree of parasitic specialization in *Phytophthora* of cacao can be evaluated by studying their ability to infect a range of differential plants to be specified. We used as a starting point the research already done in this field by Koffi Dongo BABACAUH in Ivory Coast (5) and by KOHLER of the ORSTOM team at Brazzaville (7).

1 - Plant material

An initial list (cf. annex) of egg-plant, melon, roselle and cotton plant was drawn up as plants clearly receptive, but to differing degrees, to the majority of our *Phytophthora* strains*. These plants were grown from seed on sterile vermiculite moistened with sterile distilled water. Sowing was staggered so that the different species would have reached a suitable stage when infection took place.

2 - Method of infection and interpretation of symptoms

Cultures of *Phytophthora* strains are made on liquid V8 (80 ml per flask kept in the dark for 20 days at a temperature of 25°C). The inoculum is a mycelium culture obtained by putting 10 g of fresh mycelium in 100 ml of sterile distilled water through the grinder at 10 000 revs. per minute. 1 ml of this undiluted mixture is deposited at the collar of each plant, which is then earthed up round the base of the stem with vermiculite to prevent drying out of the inoculum (7).

Several criteria for pathogenic strength evaluation were used :

- the mortality rate (or survival rate)

- number of plants showing necrosis (it will be seen in this context that the "alcohol test", which reveals a brown area as a sign of reaction, makes it possible to see the penetrated areas).

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* Thanks are due to Dr. Azaré Nyako for his cooperation in carrying out this part of our programme.

- the length of the stem (hypocotyl and epicotyl).

- the dry weight of the root-formation.

- the dry weight of the serial parts.

Depending on the reaction of the plant, one, two or more of these criteria may be used.

3 - Results and conclusions

The mortality rate results are given in Table 3. The three strains, Brazilian, Ghanaian and Cameroonian, react very differently to the various species : G_7 is highly aggressive towards all the plants while YN only attacks them mildly.

Table 3 here

The egg-plant seems to be a good differential plant enabling a clear rating of these three strains to be made. Despite high sensitivity to the two strains B_1 and G_7 from the first week onwards, the melon does make it possible to distinguish differences in virulence between these two strains. The two varieties of cotton react in identical fashion to G_7 (or YN) but differently to B_1.

Graphs 5, 6 and 7 here

The results obtained from a range of differential hosts should provide data on the greater or lesser degree of parasitic specialization of the *Phytophthora* strains, but also on the means and on the effects of attack. In fact there seems to be a correlation between mortality rates and a growth reduction in weight and in volume of the aerial parts (example given by the melon). This growth reduction is therefore a criterion not to be overlooked, particularly with plants showing no mortality (example the tomato and the capsicums). In addition, the alcohol test should be systematically applied to all survivors in order to find out which of them have been attacked and have reacted to that attack (for example with the Roselle) where there has been a defensive reaction, the inoculation spot shows a clearly visible brown ring when dipped into ethanol at 95° gl.

Finally, the infecting technique must be simple. Although inoculation at the collar seems valid, it is also feasible to incorporate the paste into the substratum or to infect directly the various plant organs in order to distinguish between the degree of parasitic specialization of the strains on the different tissues of the test plant.

(V) *GENERAL CONCLUSIONS*

The results persuade us that the most satisfactory inoculum for use in systematic study of cacao *Phytophthora* aggressiveness on organs other than the pods or on plants other than cacao is a mycelium culture which can be obtained and used under well standardized conditions.

Research work on development of infection tests has produced very important data on the possibilities of distinguishing between cacao *Phytophthora* from their pathogenic strength. It appears that the three cacao *Phytophthora* strains studied here, one from Brazil (B_1), one from Ghana (G_7) and the third from Cameroon (YN), are clearly distinguishable from one another by their attacking strength in relation to the various plants to which they have been introduced, which may point to a very different parasitic specialization in these three organisms. In particular, one can say that the Cameroonian strain which attacks the various plant species studied here only midly, is a parasite more specific to the cacao tree than are the other two strains.

Our current research covers a greater number of isolates and is tending to show that *Phytophthora* taken from West Africa are not very specialized whereas those from Cameroon and America are more diversified, some being highly specialized, others more polyphagous.

But the study goes further ; in fact, it gives rise to speculation that the Cameroonian strain, highly aggressive under natural conditions towards cacao pods, is very narrowly specialized to these pods alone and not to other organs of the plant, particularly the roots, which it attacks to a lesser degree than do the other two strains. Should it be concluded from this that very pronounced specialization of a *Phytophthora*, not only on the cacao tree but on the pods themselves, is a proper criterion for specific distinction, making it possible to anticipate the potential severity of attacks in the field ? This is what the rest of our study intends to clarify, by relating pathogenic strength vis-à-vis the cacao tree and parasitic specialization in particular to other criteria, morphological, physiological and above all cytological, such as the number and size of chromosomes (6), which are among the most important criteria in the distinction of species.

Number of days after infection / Strains	BY ROOT IMMERSION			AFTER INCORPORATION INTO THE SUBSTRATUM		
	7th day	11th day	14th day	7th day	11th day	14th day
G7	98	100	100	99	100	100
B1	86	91	92	85	93	94
YN	20	49	56	5	22	50

Table 1 : Mortality percentages of *Amelonado* seedlings infected by zoospore suspension

Number of days after infection / Strains	LOCALIZED APPLICATION at the "Collar"			GENERALIZED APPLICATION to the root system		
	7th day	11th day	14th day	7th day	11th day	14th day
G7	55	93	100	90	98	100
BI	0	5	5	23	30	30
YN	0	0	0	0	10	15

Table 2 : Mortality percentages of *Amelonado* seedlings infected by a mycelium mixture.

Table 3 : Mortality rate evolution obtained with 3 strains :

G_7 - B_1 - YN

Strains	Test Plants	6th day	8th day	10th day	12th day	14th day	16th day	18th day	20th day	22nd day
G7	Egg Plant	40	50	70	80	90				
	Melon	11	17	28	50	83	95	100	100	100
	Roselle	0	0	0	0	10		20	20	20
	SRI Cotton	53	63	74			100			
	Glandless Cotton	50	64	71			88			
B1	Egg Plant	0	15	35	55	65	68	70	70	70
	Melon	61	67	70	72	72	74	74	75	75
	Roselle	0	0	0	0	0	10		15	15
	SRI Cotton	15	24	35		53	60			
	Glandless Cotton	18	29	29		29	29			
YN	Egg Plant	0	0	0	10	20	30	40	45	50
	Melon	0	0	0	0	0	0	10	15	15
	Roselle	0	0	0	0	0	0	0	5	15
	SRI Cotton	18		30			38			
	Glandless Cotton	27		30			33			

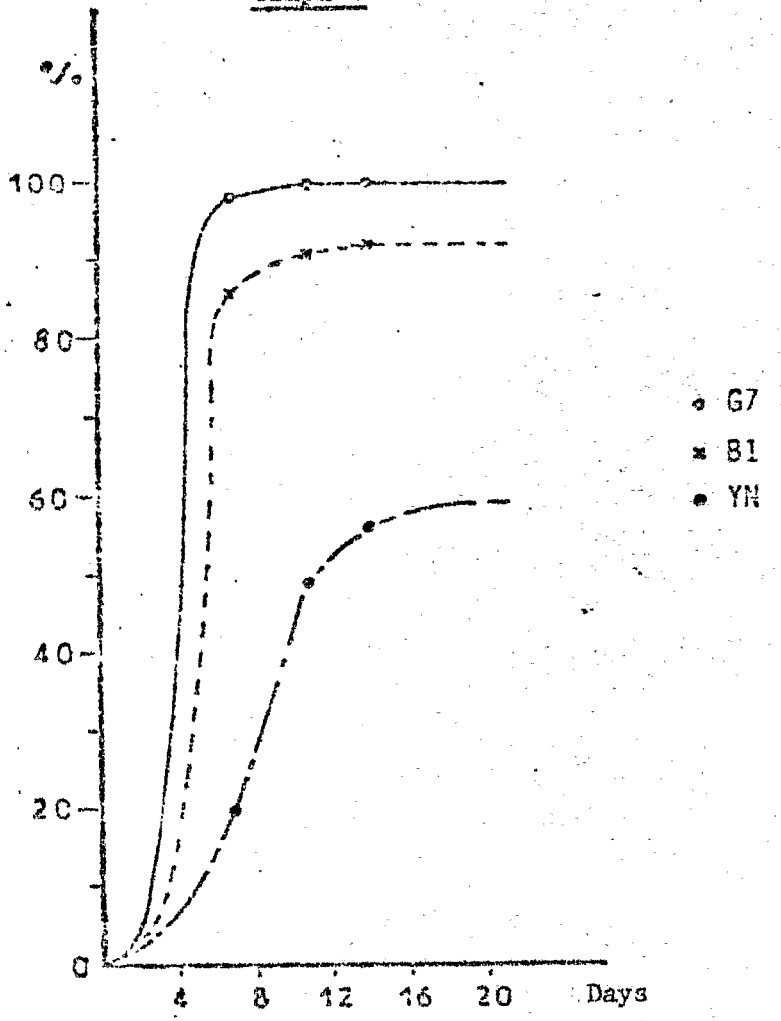
ANNEX

Solanaceae	Egg Plant, long, purple	(Tezier)
	Tomato, Marmande	(Tezier)
	Sweet Pepper	(Tezier)
	Cayenne Pepper	(Tezier)
Cucurbitaceae	Melon, Canteloup, Charentais	(Tezier)
Papilionaceae	Bean, dwarf, mangetout	(Tezier)
	Coconut, white, early	
	Bean, dwarf, mantetout black, La Victoire	(Sanrival)
	Pea, dwarf, sweet	(Sanrival)
	Soya - Kent - Group IV	(IRAT - Montpellier)
Malvaceae	Cotton plant SR_1F_4	(IRCT - Montpellier)
	Glandless 280	(IRCT - Montpellier)
	Roselle THS 22	(IRCT - Montpellier)
	Kenaf 129	(IRCT - Montpellier)

Composition of V8 medium

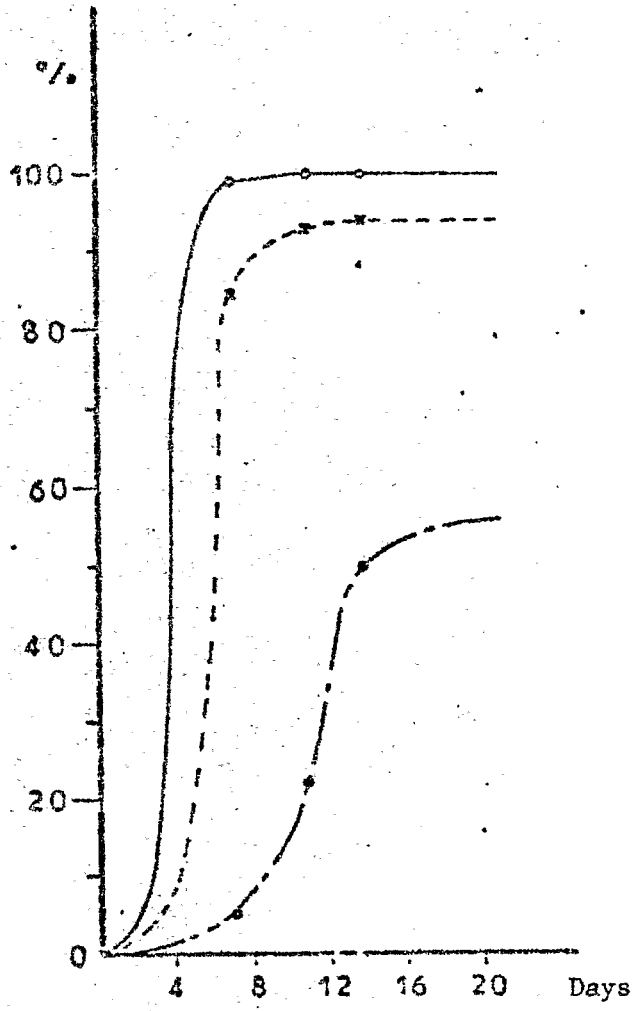
V8 of Campbell Soup Co. (1 can)	355 ml
Calcium carbonate	6.0 g
Agar-agar	30.0 g
Distilled water	2.000.0 ml

Graph 1



Immersion of roots in the suspension

Graph 2

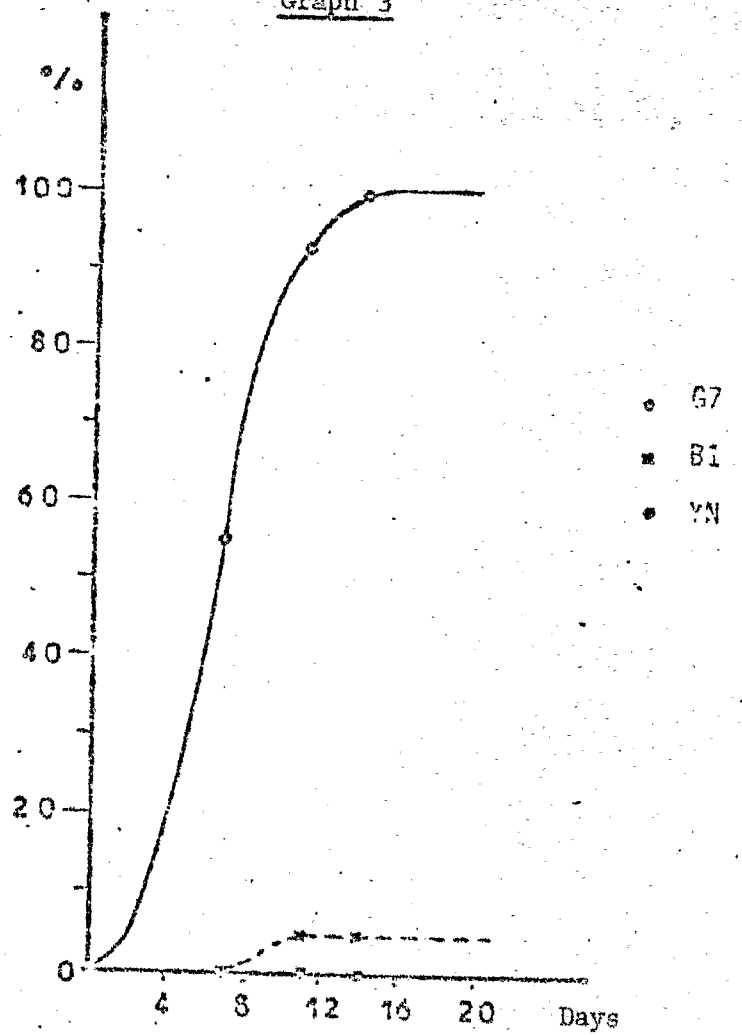


Incorporation of the suspension into the substrat

5

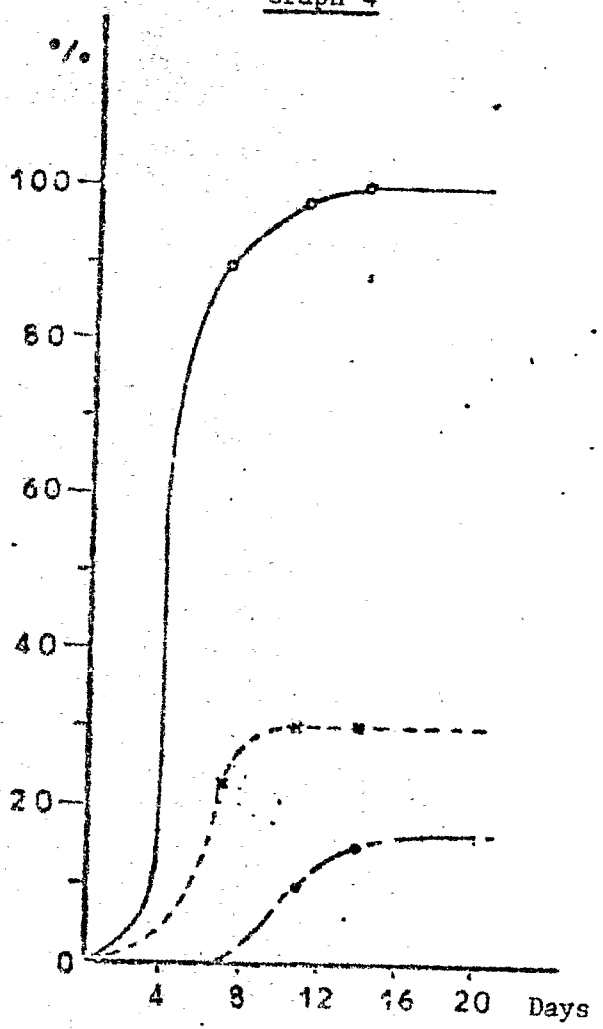
ZOOSPORE INFECTION

Graph 3



Localized applications to the collar

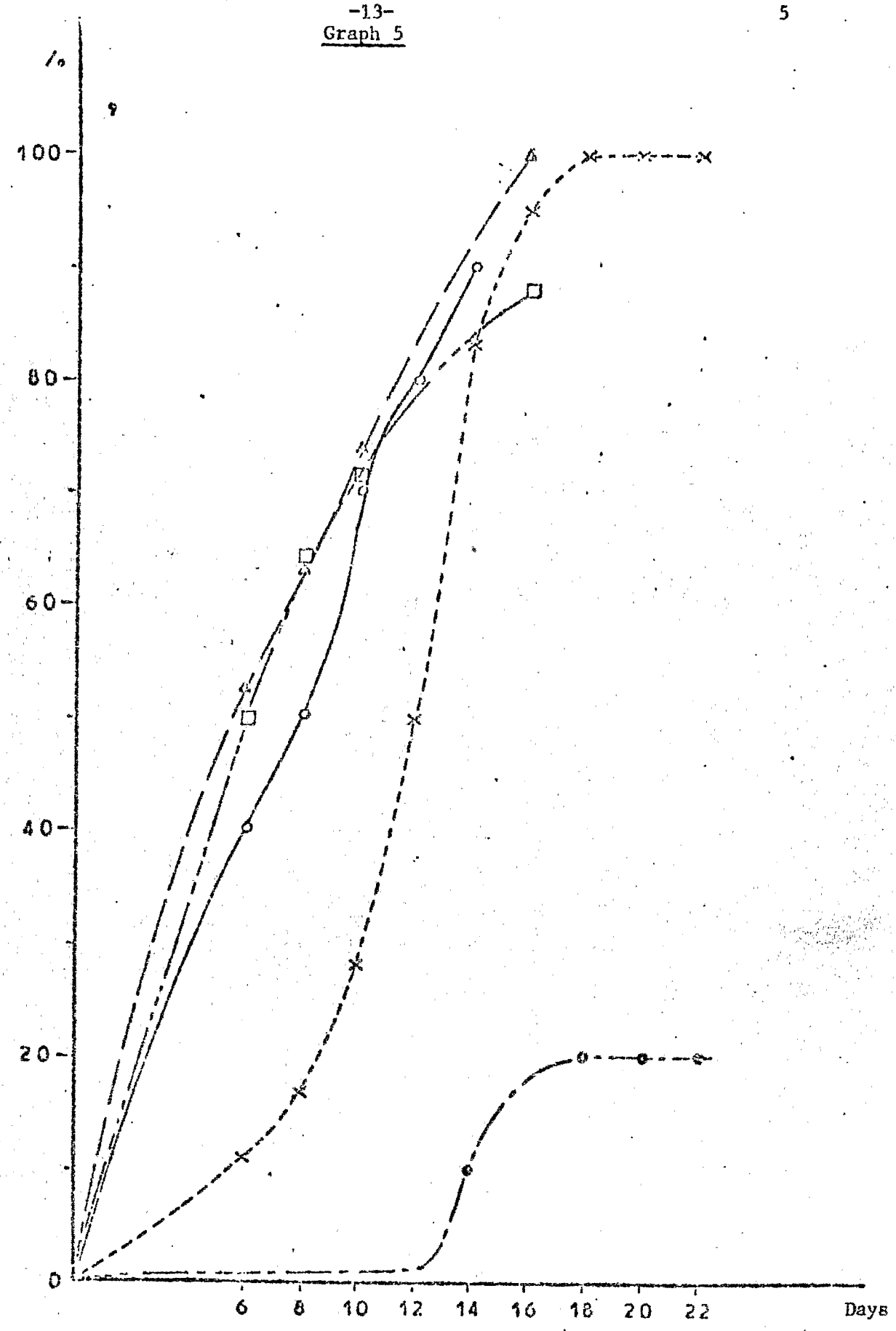
Graph 4



Generalized applications to the roots
(Incorporation into the substrate)

MYCELIUM PASTE INFECTION

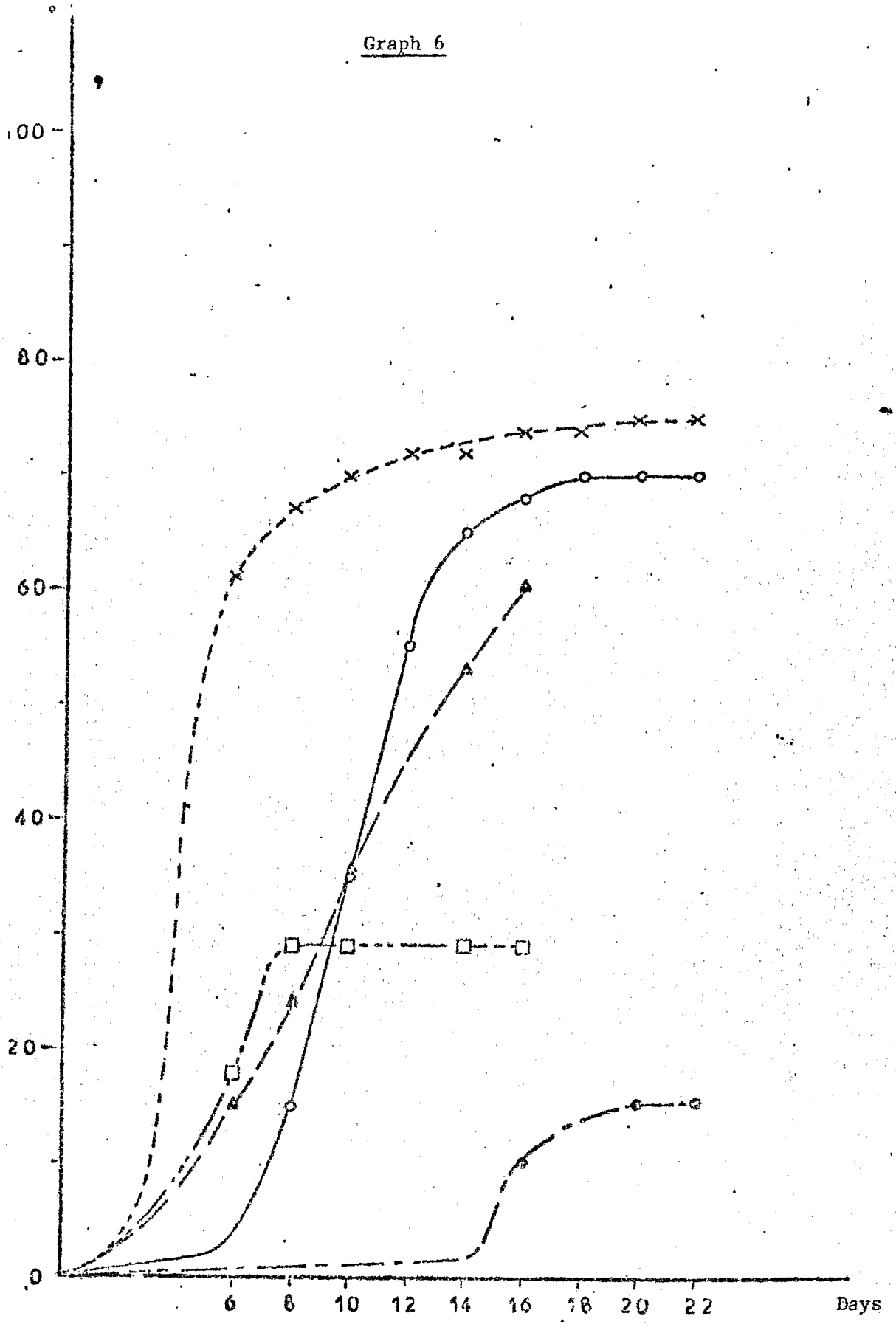
Graph 5



o Egg Plant
× Melon
o Roselle
Δ SRI Cotton
□ Glandless Cotton

MORTALITY RATE WITH C7 STRAIN

Graph 6



) Egg Plant

(Melon

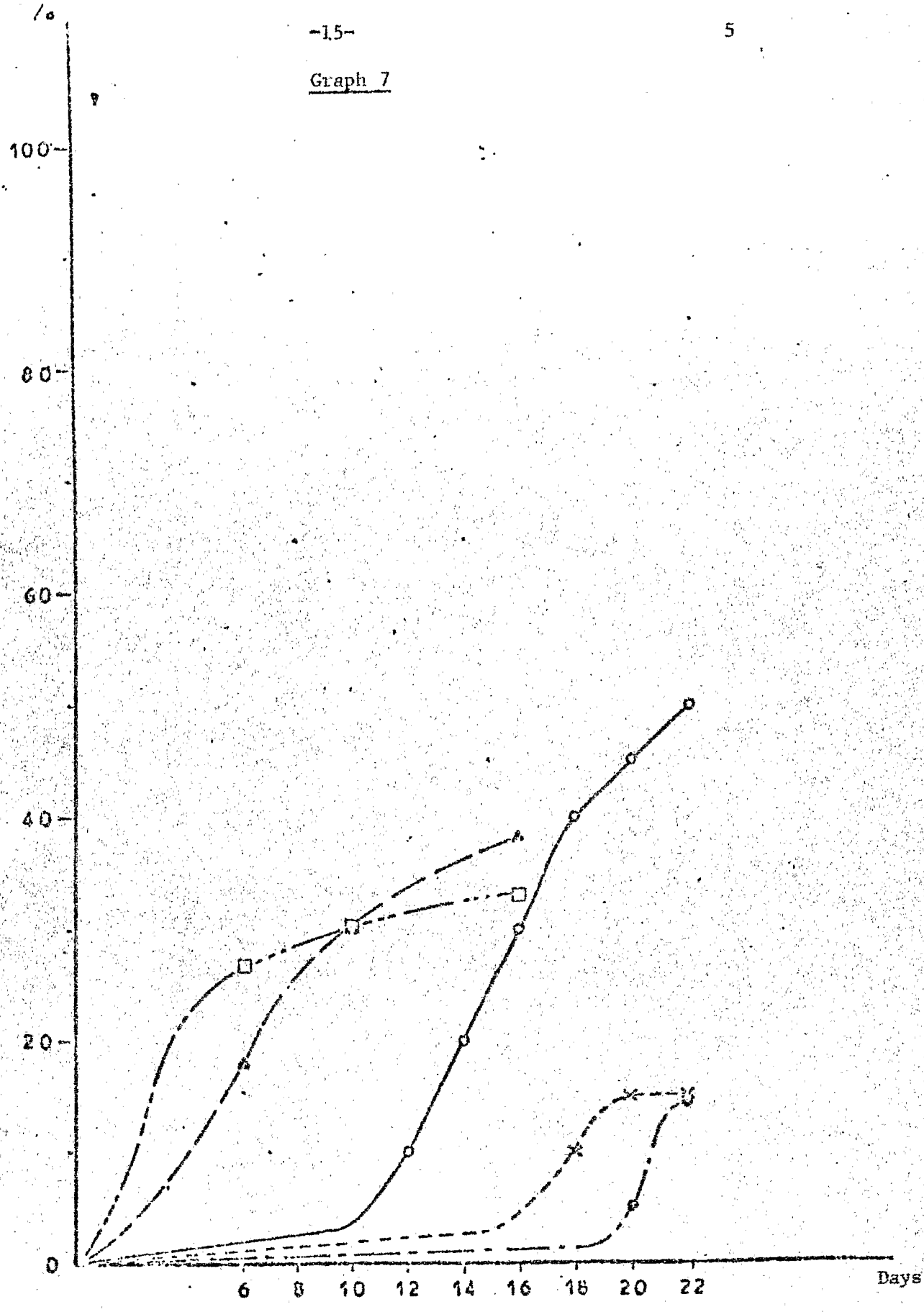
, Roselle

, SRI Cotton

) Glandless Cotton

MORTALITY RATE WITH B1 STRAIN

Graph 7



o Egg Plant
× Melon
o Roselle
Δ SRI Cotton
□ Glandless Cotton

MORTALITY RATE WITH YN STRAIN