PATHOGENIC STABILITY OF ALTERNARIA LONGIPES (ELL. & EV.) MASON SUBJECTED TO DIFFERENT METHODS OF ISOLATION, STORAGE AND INOCULUM PRODUCTION

by

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(with 1 fig.)

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Mycological methods frequently used to obtain pure, sporulating isolates of plant pathogenic fungi involve relatively long periods of artificial culture and therefore provide an opportunity for adaptive changes in the pathogen to take place. Adaptation to saprophytic conditions is often accompanied by a decrease or loss of pathogenicity in poorly specialised facultative pathogens of the *Alternaria longipes* type (VAN RAMM & LUCAS, 1963). The cultural instability of these facultative pathogens is a major problem in the assessment of varietal resistance.

This paper describes a method of isolation, storage and production of inoculum of *Alternaria longipes* which eliminated detectable pathogenic variation.

METHODS AND MATERIALS

The filter paper method was compared with a standard and a subculture method of isolation and culture to ascertain the degree and rate of variation associated with each method.

Portions of the same young 5 mm brown spot lesion on the tenth leaf of a naturally infected tobacco plant (var. Hicks) were used to make isolations of *A. longipes* for the three isolation and cultural methods under investigation.

The filter paper (FP) method

A sterile, vacuum-type spore collector, constructed on the same principle as the cyclone collector (TERVET, RAWSON, CHERRY &

SAXON, 1951), was used to collect conidia from the lesion. The spore collector was fitted with a 16 mm ID test tube and a number 12 hypodermic needle as the collector nozzle. An area on the perifery of the lesion was selected with the aid of stereomicroscope. The collector nozzle was brought into the microscope field and held approximately 1 mm above the conidia. A suction pressure of 30 cm Hg at the nozzle orifice was sufficient to dislodge and collect the conidia. These conidia were used to purify the isolate.

The lesion was surface sterilised with 70 % ethanol and a 2 % solution of sodium hyperchlorite and the following alternative technique was used on a portion of the lesion to make isolations of *A. longipes*. This technique may be used to make isolations from non- or poorly sporulating lesions.

One third of the lesion was removed, homogenised in 0.5 ml sterile, distilled water, dispensed aseptically into a test tube and its volume made up to 10 ml with distilled water. The diluted homogenate was filtered through a double layer of cheesecloth and the filtrate was used as inoculum to purify the isolate.

The filtrate from the filtered, homogenised lesion and the conidia collected from the lesion were diluted serially by 50 % six times. Each suspension in the dilution series was dispensed into a sterile atomiser and sprayed for two seconds onto the medium in a petri dish held vertically, three inches from the atomiser.

The medium consisted of a 9 cm disc of Whatman No. 17 filter paper onto which was dispensed 5 ml of a nutrient solution. The nutrient solution had the following composition:

Carrots,	10 mins. hot water (80° C) infusion from	20 g
Potatoes,	10 mins. hot water (80° C) infusion from	20 g
Dextrose,		1 g
Distilled water,	to make up	1000 ml

The petri dishes were incubated at 25° C in the dark. Colonies with a diameter of 1 mm to 2 mm arising from single conidia or hyphal fragments sporulated within 36 hours. Mycelial profileration ceased after conidial production commenced and the increase in diameter of colonies to 5—7 mm after 4 days was the result of conidiophore branching only.

After 4 days one or more petri dishes inoculated with suspensions in the dilution series had colonies separated widely enough from contaminating and other *A. longipes* colonies to allow conidia produced by a single-conidium colony to be collected with the fine-nozzled vacuum spore collector. These conidia were used to produce the source inoculum.

An exactly similar medium and spray inoculation technique were used to produce the source inoculum. The more concentrated spore suspension, however, gave a heavy, even production of conidia over the entire surface of the medium in three days. The conidia from each petri dish were harvested separately in collector test tubes. To

expedite the collection of conidia over the relatively large petri dish area a wide angled cone was fitted to the tip of the collector nozzle.

Each test tube containing the source inoculum was plugged with a rubber bung into which a tight fitting evacuation tube had previously been fitted. The rubber bung and the rim of the test tube were coated with melted paraffin wax and the evacuation tube was fitted to one of the suction points on a vacuum drying assembly. The tubes were evacuated for 30 min. at 50 cm Hg after which the evacuation tubes were sealed with flame. The evacuated tubes of source inoculum were stored at 1—2° C.

After an initial storage period of 30 days the source inoculum was used to produce bulk inoculum every two weeks for eight weeks for pathogenicity tests. Bulk inoculum was produced in the same way as source inoculum.

Standard Method

A 2 mm square portion was cut from the surface sterilised lesion referred to previoulsy, plated onto acidified 2 % PDA and incubated at 25° C. After one week the contaminated isolate was purified by mycelial subculture. This subculture was incubated for one week to verify that it was not contaminated. It was then subcultured onto a 2 % PDA test tube slant and incubated for a further week to allow sufficient growth to cover the surface of the slant. The slant was covered with sterilised paraffin oil and stored at 1—2° C.

Inoculum was produced every two weeks for 8 weeks by inoculating 2% PDA in petri dishes with hyphal fragments from the stored slant culture, and incubating them at 25° C.

Subculture Method

A third isolate was made from the surface sterilised lesion, plated onto acidified 2 % PDA and purified by mycelial subculture. Thereafter the purified isolate was subcultured weekly onto 2 % PDA and incubated at 25° C in the dark for eight weeks. After the second, fourth, sixth and eighth week additional subcultures were made onto 2 % PDA to produce bulk inoculum for pathogenicity tests.

The subculture made for inoculum production after the eighth week failed to sporulate in twenty days. In this case the inoculum for pathogenicity tests was made by scraping the mycelium from the surface of the 20 day old culture and homogenising it in distilled water.

Plant Inoculation and Disease Rating

Each of the inocula were suspended in distilled water and the concentration of conidia adjusted to 3×10^4 conidia/ml. The leaves of ten week old tobacco plants, var. Hicks, were inoculated using a fine atomised spray. The atomised inoculum was sprayed onto individual leaves until incipient runoff. Ten plants were inoculated with each of the inocula tested.

The inoculated tobacco plants were transferred to, and retained for 10 days in a humidity chamber at ± 100 % RH with a diurnal temperature range of 23—26° C and a light intensity at 12 noon of 800 l/sq. ft. Afterwhich the plants were replaced on the greenhouse table at a RH of 65—75 %, a diurnal temperature range of 23—26°C and a noon light intensity of 1000 l/sq. ft. On the fourteenth day after inoculation the plants were rated for disease severity according to the following scale:

0 No lesions.
1 Few 1—2 mm diameter light-brown lesions restricted to the lower leaves.
2 Numerous 1—2 mm diameter dark brown lesions with faint chloratic halos mainly limited to the lower leaves.
3 Large 3—5 mm diameter lesions restricted to lower leaves with a few small scattered light-brown lesions on the upper leaves.
4 Large 4—6 mm diameter lesions on the lower and upper leaves.

Plants showing intermediate reactions were given 0.5 value disease ratings.

Results and Discussion

The results of the pathogenicity tests (Fig. 1) confirm the proposal based on the findings of Van Ramm & Lucas (1963) that a prolongation of vegetative (saprophytic) growth of *A. longipes* in culture would cause a shift towards the saprophytic state with a consequent decrease in pathogenicity.

The filter paper (FP) technique described in this paper reduced the total period of culture from isolation to bulk inoculum production to 9 days compared with 34 days in the standard method. Furthermore at least two-thirds of the cultural period in the FP technique was devoted to sporulation and branching of the aerial conidiophores. This reduction in period of vegetative growth was apparently instrumental in eliminating variation in pathogenicity. A major proportion of the prolonged cultural period of the standard method was devoted to mycelial proliferation which provided an opportunity for adaptive mechanisms to cause degenerative physiological changes.

An extension of the phase of vegetative growth coupled with repeated mycelial subculturing caused pronounced attenuation in *A. longipes* and the development of total saprophytism and a loss of sporulative ability.

Attenuation and the loss of the ability to sporulate which accompanies repeated mycelial subculturing is due to the loss or repression of genetic determinants. Inactivation or a loss of genes according to Jinks (1956) is due to the different degree of completeness of extranuclear genetic determinants required for the formation of mycelia and conidia. The repetitive use of genetically unbalanced mycelium

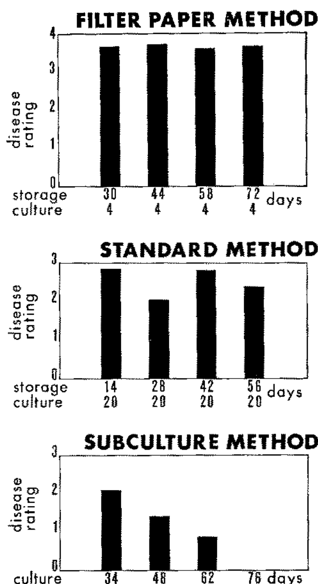

Fig. 1. Histograms of the mean disease ratings of ten week old tobacco plants inoculated with *A. longipes* which had been subjected to different methods and lengths of storage and culture.

would thus progressively 'select out' sporulation determinants. The progressive loss of pathogenicity during this selection process suggests that genetic factors governing these phenomena are linked to a single pleiotropic cytoplasmic determinant (JINKS, 1956). Cytoplasmic determinants need not be evoked to account for degenerative forms of adaption to the cultural environment. Dissociation of heterokaryons into morphologically and physiologically dissimilar homokaryotic components is a well known phenomenon (PARMETER, SNYDER & REICHLE, 1963).

A selection pressure advantage given by the cultural environment to one homokaryon would result in a progressive change in the characteristics of the culture to those of the homokaryon with a selection advantage.

Somatic dissociation of a heterokaryon is not necessary for the effective operation of selection pressure in changing the characteristics of a culture (BEADLE & COONRAADT, 1944). The nuclear ratio in a "wild type" isolate may change, due to a selection advantage given to division rate of one or more of the nuclear components, and thus alter the phenotype of the heterokaryon to one which is in equilibrium with the cultural environment.

A third possible mechanism which validly explains the ability of *A. longipes* to shift from a pathogenic to saprophytic state is a progressive repression of genes governing the metabolic machinery nec-

essary for pathogenicity (and sporulation) in the cultural environment. An accompanying progressive derepression of genes responsible for the synthesis of enzyme systems necessary for the more efficient utilisation of the cultural substrates would enhance the degree of saprophytism. A necessary corollary to account for the permanent loss of pathogenicity and sporulation is that the greater the prolongment and degree of repression the less is the likelihood of derepression occurring to re-establish pathogenic and sporulation capabilities of the fungus.

The explanation for the instability of *A. longipes* and similar facultative parasites under cultural conditions is a moot point. The prolongation of vegetative growth in culture, however, appears essential if these mechanisms are to affect cultural variation. From the plant pathological point of view, if the isolation, storage and sporulation can be achieved with minimal vegetative growth relative stability of the pathogen can be insured.

The filter paper method may be used with minor modifications to prepare inoculum for large scale greenhouse screening and field variety trials. Preliminary results (not reported) show that inoculated filter paper or thick blotting-paper discs may be air-dried after a week of incubation, and ground to a dust in a mill. The cellulose dust serves as an inert carrier for the spore inoculum. The inoculum can be dusted onto plants in the greenhouse or field with a conventional pesticide duster.

Summary

The pathogenic stability of *A. longipes* was greatest when the composition of the medium promoted maximum sporulation and minimal mycelial proliferation.

A Whatman No. 17 filter paper disc saturated with an 0.1 % dextrose infusion medium from carrots and potatoes minimised mycelial proliferation, and promoted rapid and extensive spore production in two to four days at 25° C. Approximately 75 % of the cultural period on 2% PDA was devoted to mycelial proliferation. The difference in extent of mycelial growth in the filter paper and standard methods was apparently instrumental in eliminating a decline in pathogenicity when using the former method. Weekly mycelial subculturing on 2 % PDA caused rapid drop in pathogenicity and a total loss of pathogenicity and sporulative ability between the 62nd and 76th day.

The use of a modified filter paper method for large scale inoculum production for greenhouse and field variety trials is discussed.

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