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Effect of inoculum composition on infection of French bean leaves by conidia of Botrytis cinerea

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Abstract

Inoculation of leaves of French bean *(Phaseolus vulgaris)* with sprays or small drops of a suspension of conidia of *Botrytis cinerea* gave rise to spreading lesions, lesions remaining restricted in size or to no visible necrosis. The type of reaction depended on the composition of the inoculum.

In studies with drop inoculations with buffered inocula some of the factors involved were analyzed. The formation of spreading lesions depended on pH, type and molarity of the buffer, presence of glucose, and concentration of conidia in the inoculum. If the phosphate buffer used in most of the inocula was replaced by monobasic phosphate, similar results were obtained. The reactions were not influenced by the proportion of K^+ or Na^+ ions in the phosphate buffer.

Inoculations with conidia suspended in a solution of 0.067 M phosphate buffer (pH 5.0) or monobasic phosphate and 0.11 M glucose always evoked a susceptible reaction, i.e. the formation of spreading lesions.

Additional keywords: Phaseolus vulgaris, phosphate buffer, inorganic phosphate, glucose, spreading lesions.

Introduction

Infection of many host plants by *Botrytis cinerea* Pers. ex Nocca & Balbis starts from moribund tissues colonized by this pathogen (Jarvis, 1977; Verhoeff, 1980). Only a few reports describe briefly how *B. cinerea* infects (dwarf) French bean plants *(Phaseolus vulgaris* L.*)*. in the field under natural conditions (Campbell, 1949; Hubbeling, 1955; Zaumeyer and Thomas, 1957). Infection usually occurs where an old blossom has fallen on a leaf or other plant part or has been retained at the tip of the pod, or where discarded cotyledons remain in contact with the hypocotyl. Under moist conditions conidia of *B. cinerea* can germinate and mycelium can develop on those withered or dead plant parts. Such colonized tissues are used by the fungus as a saprophytic base from where infection in the underlying living tissues can start. Infection is favoured on plant parts damaged by certain viral, bacterial or fungal pathogens, by frost or hail, or by some other causes.

Infection by mycelium growing from a dead plant substrate could be mimicked by inoculation with agar disks supporting mycelial growth or with mycelium in nutrient solution (Polach and Abawi, 1975; Van den Heuvel, 1976; Van den Heuvel and

Grootveld, 1978; Wasfy et al., 1978; García-Arenal and Sagasta, 1980). Spreading lesions in unwounded healthy bean tissue were also obtained after inoculation with conidia suspended in 1% orange juice or in Czapek broth, but not with conidia in distilled water without added nutrients (Dixon and Doodson, 1975; García-Arenal and Sagasta, 1980). Apparently, successful infections are dependent on factors that stimulate conidial germination and further development of *B. cinerea* directly or via interaction with co-occurring micro-organisms, or that, alternatively, predispose plant tissue to infection.

The present paper describes some factors which determine a susceptible reaction, i.e. the formation of spreading lesions, in unwounded bean leaves after inoculation with conidia of *B. cinerea*. A preliminary report has already appeared (Van den Heuvel, 1980).

Materials and methods

Growth of B. cinerea and bean plants. Sporulating cultures of *B. cinerea* (isolate BC-1) (Van den Heuvel, 1976) were obtained after growing the fungus on 25-mm-wide PDA slants at about 23 °C under continuous white fluorescent light (1750 lx). Twelve-to-sixteen-day-old cultures were flooded with distilled water and the conidia were scraped from the cultures. The suspension was filtered through a thin layer of glass wool and the filtrate was centrifuged (1400 *g*, 10 min). The conidia in the pellet were resuspended in one of several buffer and nutrient solutions to be tested. The final concentration was, unless stated otherwise, 2×10^6 conidia.ml^{-1}.

French bean (cv. Dubbele Witte zonder draad) plants were grown in the glasshouse at 22 ° to 27 °C and used for inoculation when 10 to 13 days old.

Spray inoculations. Plants were sprayed with conidial suspensions until drops started to run off from leaves. Inoculated plants were incubated in the glasshouse at 17 ° to 20 °C under conditions of high relative humidity (in humidity chambers lined with moistened filter paper).

Drop inoculations. Detached primary leaves were placed on perforated plastic grids on a layer of wet cotton-wool in transparent plastic trays. The grids kept the leaf blades separated from the cotton-wool, but care was taken that the cut end of each petiole made contact with the cotton-wool. Six 5-μl drops of condidial suspension were placed on the adaxial side of each of five leaves per treatment. Trays containing ten inoculated leaves were enclosed in transparent plastic bags and incubated at 19 °C and a 16 h photoperiod (fluorescent light, 1200 lx). At various times after inoculation numbers of spreading lesions were determined.

All experiments were repeated at least once. From each set of duplicate experiments with similar results, only results of one experiment are given.

Results

Symptoms after spray and drop inoculations. On most primary and a few trifoliate leaves of glasshouse-grown bean plants sprayed with *B. cinerea* conidia (2×10^4 or 2×10^5 conidia.ml^{-1}) in a modified Richards' solution, with 0.11 M glucose as the only carbon source, several small, necrotic lesions were formed that remained

restricted in size (diameter less than 2 mm), but some of them developed into spreading lesions. Inoculations with conidia in water (2×10^4 or 2×10^5 conidia.ml^{-1}) yielded a few small, necrotic lesions only; even 15 days after inoculation no spreading lesions developed. Sprays with water or Richards' solution only did not induce the formation of lesions.

Fig. 1. Spreading (S) and small, non-spreading (NS) lesions five days after spray inoculation of primary bean leaves with 10^5 *B. cinerea* conidia per ml of 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) + 0.11 M glucose.

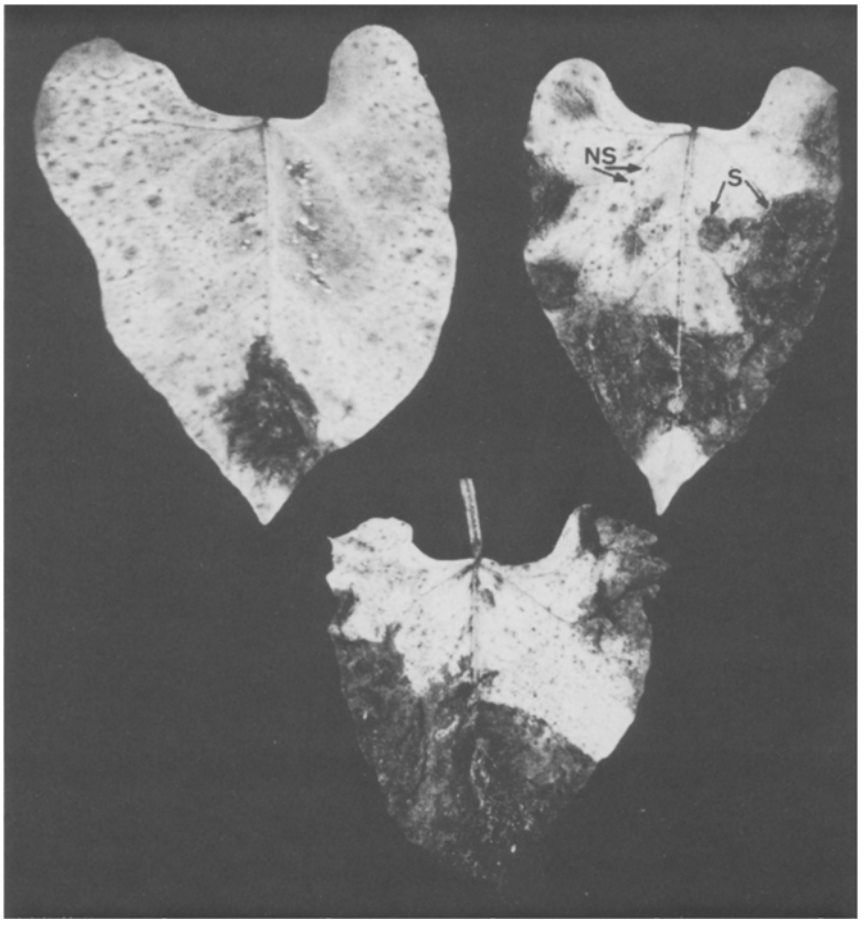

Fig. 1. Zich uitbreidende (S) en kleine, zich niet uitbreidende (NS) lesies vijf dagen na inoculatie d.m.v. besproeiing van primaire bonebladeren met 10^5 B. cinerea-conidiën per ml 0,067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5,0) = 0,11 M glucose.

Spray inoculations of detached primary leaves of 10-day-old glasshouse-grown beans with conidia (10^5 or 10^6 conidia.ml^{-1}) suspended in 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) + 0.11 M glucose also gave rise to many small and a few spreading lesions. No relation of spreading lesions with certain sites of the leaf was observed (Fig. 1).

Non-spreading and spreading lesions could also be obtained after inoculation of leaves with single small drops of conidial suspensions. This pointed to the use of a standardized inoculation method and an incubation under controlled conditions (both described under Materials and methods) with which the effects of several factors on the incidence of spreading lesions were investigated.

In these experiments non-spreading lesions were lesions that remained restricted in size, consisting of a superficial necrosis on part or all of the area under the inoculation drop (max. diam 4 mm), and sometimes extending to the abaxial side of the leaf. Often the lesions consisted only of a number of brown epidermal cells. Only those lesions that spread beyond the edge of the inoculum drop and that had also extended to the abaxial side of the leaf, were regarded as spreading lesions. Most spreading lesions started spreading on the second day after inoculation, and their subsequent growth was almost linear (Fig. 2).

pH and type of buffer solution. Drop inoculations with conidia suspended in 0.1 M citric acid-Na_2HPO_4 buffer + 0.11 M glucose or 0.067 M KH_2PO_4-Na_2HPO_4 buffer + 0.11 M glucose were effective in inducing spreading lesions only at or below pH 6.5 (Fig. 3). At higher pH mostly non-spreading lesions or no lesions at all were formed. The formation of spreading lesions was not only dependent on pH, but also on the type of buffer in the inoculum, as only few spreading lesions developed after inoculation with conidia in 0.1 M Na-acetate-acetic acid buffer + 0.11 M glucose.

In control experiments with the three buffers (at pH 5.0) + glucose, but without conidia, drops of 0.1 M citric acid-Na_2HPO_4 buffer + 0.11 M glucose, but not of the two other buffer solutions, sometimes caused some visible necrosis below the drops. Further experiments revealed that necrosis was caused by citric acid, in particular at pH below 5.0.

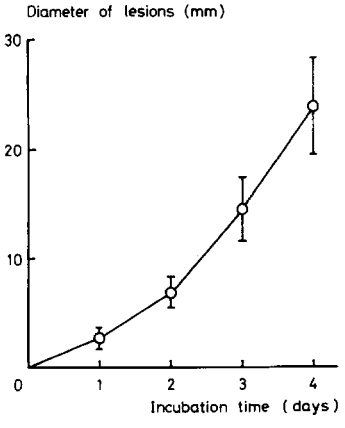

Fig. 2. Diameter of spreading lesions at different times after inoculation of leaves with 5-µl drops of an inoculum containing 10^4 conidia per 5 µl of 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) + 0.11 M glucose. Vertical lines denote standard deviations from means obtained from 30 lesions measured in two directions.

Fig. 2. Diameter van zich uitbreidende lesies op verschillende tijdstippen na inoculatie van bladeren met 5-µl druppels van een inoculum met 10^4 conidiën per 5 µl 0,067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5,0) + 0,11 M glucose. Verticale strepen geven de standaarddeviatie weer van de gemiddelden van 30 lesies die in twee richtingen gemeten zijn.

Subsequent experiments were carried out with conidia suspended in KH_2PO_4-Na_2HPO_4 buffer supplemented with glucose.

Fig . 3. Numbers of spreading lesions four days after inoculation of leaves with series of 30 5-μl drops of inocula containing 10^4 conidia per 5 μl of solutions of three different buffers of varying pH's and supplemented with glucose.
○ = 0.1 M citric acid-Na_2HPO_4 buffer + 0.11 M glucose;
△ = 0.1 M Na-acetate-acetic acid buffer + 0.11 M glucose;
□ = 0.067 M KH_2PO_4-Na_2HPO_4 buffer + 0.11 M glucose.

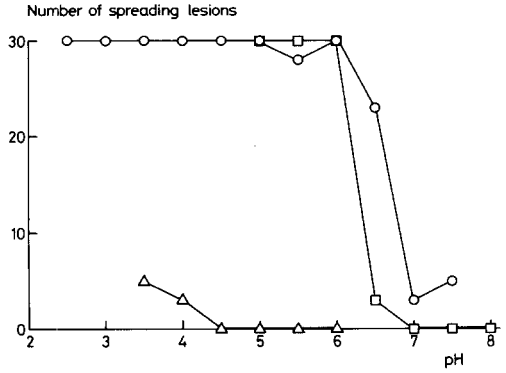

Fig. 3. Aantallen zich uitbreidende lesies vier dagen na inoculatie van bladeren met series van 30 5-μl druppels van inocula met 10^4 conidiën per 5 μl van oplossingen van drie verschillende buffers met variërende pH's en aangevuld met glucose.
○ = 0,1 M citroenzuur-Na_2HPO_4 buffer + 0,11 M glucose;
△ = 0,1 M Na-acetaat-azijnzuur buffer + 0,11 M glucose;
□ = 0.067 M KH_2PO_4-Na_2HPO_4 buffer + 0,11 M glucose.

Fig. 4. Numbers of spreading lesions at different times after inoculation of leaves with series of 30 5-μl drops of inocula containing 1 to 10^4 conidia per 5 μl of 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) + 0.11 M glucose

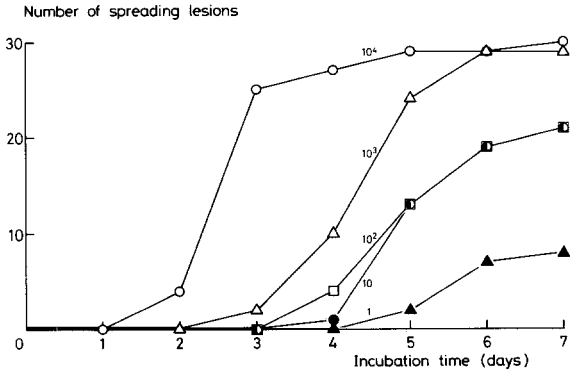

Fig. 4. Aantallen zich uitbreidende lesies op verschillende tijdstippen na inoculatie van bladeren met series van 30 5-μl druppels van inocula met 1 tot 10^4 conidiën per 5 μl 0,067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5,0) + 0,11 M glucose.

Concentration of conidia. The formation of spreading lesions was dependent on the concentration of conidia suspended in 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) + 0.11 M glucose (Fig. 4). Rate of appearance and final numbers of spreading lesions were positively correlated with concentration of conidia. Even one conidium per inoculum drop gave sometimes rise to a spreading lesion.

Molarity of phosphate buffer. Fig. 5 shows that the development of spreading lesions was also dependent on the molarity of the KH_2PO_4-Na_2HPO_4 buffer in the inoculum. At molarities below 0.02 M no (in this experiment) or only few (in other experiments) spreading lesions were formed.

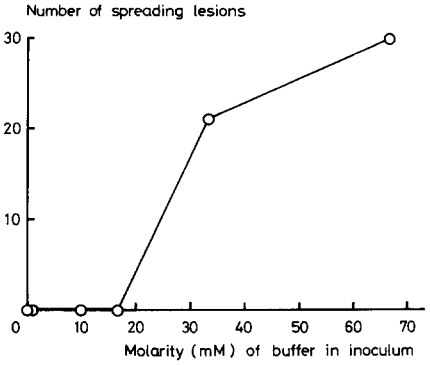

Fig. 5. Numbers of spreading lesions three days after inoculation of leaves with series of 30 5-µl drops of inocula containing 10^4 conidia per 5 µl of KH_2PO_4-Na_2HPO_4 buffer solution (pH 5.0) of different molarities supplemented with 0.11 M glucose.

Fig. 5. Aantallen zich uitbreidende lesies drie dagen na inoculatie van bladeren met series van 30 5-µl druppels van inocula met 10^4 conidiën per 5 µl KH_2PO_4-Na_2HPO_4 bufferoplossing (pH 5,0) van verschillende molariteit en aangevuld met 0,11 M glucose.

Fig. 6 Numbers of spreading lesions at different times after inoculation of leaves with series of 30 5-µl drops of inocula containing 10^4 conidia per 5 µl of 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) supplemented with 0.111 M (○), 0.027 M (△), 0.006 M (□) or 0.001 M (●) glucose or without added glucose (▲).

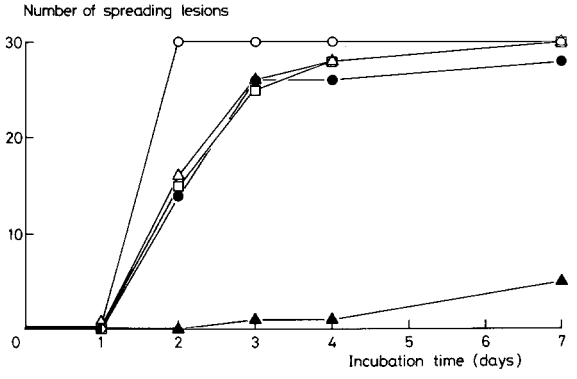

Fig. 6. Aantallen zich uitbreidende lesies op verschillende tijdstippen na inoculatie van bladeren met series van 30 5-µl druppels van inocula met 10^4 conidiën per 5 µl 0,067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5,0) aangevuld met 0,111 M (○), 0,027 M (△), 0,006 M (□) of 0,001 M (●) glucose of zonder toegevoegd glucose (▲).

Glucose concentration. Many spreading lesions were formed in leaves after inoculations with conidia in 0.067 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.0) with different concentrations of glucose (Fig. 6). Such an inoculum without glucose yielded only few spreading lesions. No spreading lesions were formed after inoculation with conidia suspended in a 0.010 M phosphate buffer (pH 5.0) with 0.001 to 0.111 M glucose or without glucose, or in a 0.111 or 0.028 M glucose solution in distilled water or in distilled water only.

Table 1. Numbers of spreading lesions four days after inoculation of leaves with series of 30 5-µl drops of inocula containing 10^4 conidia per 5 µl of 0.010 or 0.067 M phosphate buffer solutions (pH 5.0) prepared from K^+ and Na^+ salts in different combinations, and supplemented with 0.11 M glucose.

Molarity of buffer (M)	Salt composition of buffer			
	KH_2PO_4 + K_2HPO_4	KH_2PO_4 + Na_2HPO_4	NaH_2PO_4 + K_2HPO_4	NaH_2PO_4 + Na_2HPO_4
0.010	0	0	1	0
0.067	30	29	30	30

Tabel 1. Aantallen zich uitbreidende lesies vier dagen na inoculatie van bladeren met series van 30 5-µl druppels van inocula met 10^4 conidiën per 5 µl van 0,010 of 0,067 M fosfaatbuffer-oplossing (pH 5,0) bereid uit K^+ en Na^+-zouten in verschillende combinaties en aangevuld met 0,11 M glucose.

Table 2. Numbers of spreading lesions four days after inoculation of leaves with series of 30 5-µl drops of inocula containing 10^4 conidia per 5 µl of phosphate solutions of different types and concentrations, supplemented with 0.11 M glucose.

Concentration (M)	Type of phosphate			
	H_3PO_4	KH_2PO_4	KH_2PO_4 + NA_2HPO_4 (pH 5.0)	K_2HPO_4
0.001	—[a]	0	—	—
0.005	—	0	—	—
0.010	24	1	2	0
0.017	—	3	—	—
0.033	—	23	—	—
0.067	30	30	24	12

[a] — not determined.

Tabel 2. Aantallen zich uitbreidende lesies vier dagen na inoculatie van bladeren met series van 30 5-µl druppels van inocula met 10^4 conidiën per 5 µl van oplossingen van verschillende types en concentraties fosfaat, aangevuld met 0,11 M glucose.

Proportion of K^+ and Na^+ ions. Experiments were done with inocula containing phosphate buffer solutions (at pH 5.0) prepared from K^+ and Na^+ salts in four different combinations. Table 1 shows that the formation of spreading lesions with 0.067 M buffer inocula and that of non-spreading lesions with 0.010 M buffer inocula were independent of the proportion of K^3 and Na^3 ions in the inocula.

Concentrations and type of phosphate. The formation of spreading lesions was dependent on concentration and type of phosphate in the inoculum (Table 2). Solutions of KH_2PO_4 were about as effective as KH_2PO_4-Na_2HPO_4 buffer solutions (pH 5.0). Solutions of K_2HPO_4 were less effective in inducing spreading lesions, whereas inocula containing orthophosphoric acid were most effective.

Discussion

The results reported here demonstrate that the composition of the inoculum is a determining factor for the response of unwounded primary French bean leaves to conidia of *B. cinerea.* Changes in composition may lead to a change in the outcome of the host pathogen interaction. The formation of spreading lesions was dependent on concentration of conidia, pH, type and molarity of buffer, type and concentration of inorganic phosphate, and presence of glucose. Addition to the inoculum of phosphate, in particular as monobasic phosphate or orthophosphoric acid, and glucose at concentrations of 0.067 M and 0.11 M, respectively, evoked a susceptible reaction, i.e. the formation of spreading lesions.

Free or bound phosphate and glucose are very common components of primary and secondary metabolism of living organisms, and may become readily available in moribund plant tissues, e.g. discarded bean blossoms, that are colonized by *B. cinerea* prior to infection of leaves.

At this stage, the mode of action of the infection-promoting substances is unknown. They may act either directly on foliar or fungal metabolism or on the interaction between leaf and pathogen, or indirectly on growth or metabolism of co-occurring epiphytic micro-organisms. It is to be investigated which stage(s) of the infection process and which metabolic processes are affected. García-Arenal and Sagasta (1980) observed a correlation between the complexity of the penetration structure of *B. cinerea* on French bean hypocotyls and the nutritional status of the inoculum.

A factor acting directly on the metabolism of the leaves may predispose them to infection by *B. cinerea.* Phosphate may act as such a predisposing factor, since injury to bean leaves caused by phosphorus compounds has been reported. Phosphates and other P compounds have been used in foliar applications of nutrients (Burghardt, 1961; Barèl and Black, 1979). Application has been limited, however, primarily because no P compound is available which can be sprayed on plants in large enough quantities without damaging the leaves. Most compounds were not injurious to foliage at 0.05 M (Silberstein and Wittwer, 1951). The compounds most toxic to bean leaves were NaH_2PO_4 and $Ca(H_2PO_4)_2$ (Krzysch, 1958). Uptake and toxicity of orthophosphoric acid were maximal at pH below 3.5 (Swanson and Whitney, 1953). Yarwood (1952) found that injury was caused by rubbing carborundum-dusted bean leaves with dibasic phosphate (pH 8.5). On the other hand, orthophosphate,

although it was shown to be a competitive inhibitor of ornithine carbamoyltransferase, protected the bean enzyme from inactivation by phaseotoxin (Kwok et al., 1979). In the present study no visible injury to bean leaves by 0.067 M monobasic phosphate or phosphate buffer (at pH about 5.0) has been observed.

Foliar application of phosphate as a nutrient may, thus, be hazardous for two reasons: it may cause injury and it may stimulate infection by *B cinerea* and perhaps other pathogens. Addition of glucose and 0.01 M potassium phosphate buffer (pH 6.0) to the inoculum stimulated formation of spreading lesions of *B. fabae,* but not of *B. cinerea,* in broad bean leaves (Purkayastha and Deverall, 1965). Addition of phosphate to the inoculum also increased numbers of lesions in French bean leaves formed by several viruses (Yarwood, 1952), and stimulated development of soft rot in leaves of Chinese cabbage by *Erwinia carotovora* var. *carotovora* (Tanaka and Kikumoto, 1978).

The results obtained with phosphate and glucose in the inoculum warrant a further investigation into the phenomenon of infection stimulation in various host pathogen interactions. More research is in progress on the importance and rôle of phosphate and glucose in *B. cinerea* infections.

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Samenvatting

De invloed van de inoculumsamenstelling op de infectie van slaboonbladeren door conidiën van Botrytis cinerea

Inoculatie van bladeren van slaboon *(Phaseolus vulgaris)* d.m.v. besproeiing of het opbrengen van kleine druppeltjes van een suspensie van conidiën van *Botrytis cinerea* induceerde de vorming van zich uitbreidende lesies, lesies die beperkt van omvang bleven of geen zichtbare necrose. Het type reactie hing af van de samenstelling van het inoculum.

In proeven met druppel-inoculaties met gebufferde inocula werden enkele van de factoren geanalyseerd. De vorming van zich uitbreidende lesies hing af van pH, type en molariteit van de buffer, aanwezigheid van glucose en concentratie van de conidiën in het inoculum. Als de fosfaatbuffer die in de meeste inocula gebruikt werd, werd vervangen door monobasisch fosfaat, werden gelijke resultaten verkregen. De reacties werden niet beïnvloed door de verhouding van K^+ of Na^+-ionen in de fosfaatbuffer.

Inoculaties met conidiën gesuspendeerd in een oplossing van 0,067 M fosfaatbuffer (pH 5,0) of monobasisch fosfaat en 0,11 M glucose brachten altijd een vatbare reactie, d.w.z. de vorming van zich uitbreidende lesies, teweeg.

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