Temperature Responses of Isolates of *Macrophomina phaseolina* from Different Climatic Regions of Sunflower Production in Italy

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ABSTRACT

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Sixty-four isolates of *Macrophomina phaseolina* from sunflower, collected in four different climatic areas of Italy (north, mideast, south, midwest), were subjected to growth rate (GR) tests at 15, 25, 30, 35, and 40°C, and to chlorate resistance tests. The pathogenicity of 24 isolates was also tested on maize, sorghum, soybean, sunflower, safflower, sugar beet, kenaf, and melon seedlings. The optimum temperature for growth was 30°C for 62 isolates and 35°C for two isolates. Isolate GR varied considerably at all temperatures ($P < 0.01$) but the maximum variability between isolates occurred at 15 and 40°C. Isolates from the north (colder area) grew better at lower temperatures than other isolates and also showed a good adaptability to 40°C. Isolates from the midwest (Mediterranean climate) had the fastest GR at 40°C but the worst GR at the lowest temperature tested. Isolates from the mideast and south, with Mediterranean climate, grew better at the optimum temperatures (30 and 35°) and showed the poorest adaptability to the limit temperatures (15 and 40°C). Among the isolates, 95% were chlorate tolerant, 2% were chlorate sensitive and 3% were chlorate resistant; among the chlorate tolerant isolates, 47% had feathery chlorate phenotype and 48% only showed less dense colonies than the control without potassium chlorate. The isolates were very pathogenic on soybean, moderately pathogenic on sunflower, safflower, sorghum, and melon, mildly pathogenic on sugar beet and kenaf, and not pathogenic on maize using an in vitro seedling inoculation assay. These results suggest that *M. phaseolina* isolates from sunflower can adapt to continental conditions such as those of northern Italy, and that the fungal population, which is chlorate tolerant and pathogenic to seedlings of various cultivated species, can be a potentially widespread pathogen on many crops.

Additional keywords: charcoal rot, chlorate sensitivity

Macrophomina phaseolina (Tassi) Goidanich is a pathogen of a wide number of cultivated and wild species in warm, temperate, and tropical regions of the world. It causes charcoal rot (CHR), leaf blight, stem blight, and damping off. Its pathogenicity increases with a rise in temperature, with an optimum between 28 and 35°C (4). Host water stress is another principal factor favoring development of the disease (1,8).

In warmer countries where sunflower is cultivated, CHR is the most important disease (10,26,27), while in colder and wet areas, *Sclerotinia sclerotiorum* (Lib.) de Bary is the other important pathogen causing sunflower stem disease (22,24).

Although only one species is recognized within the genus *Macrophomina* (31), great variability in morphology has been observed among isolates from different hosts (20,21). Many researchers have also found great variability in pathogenicity and in morphological characteristics among isolates from the same host (3,10,30) and between isolates from different parts of the same plant (2). The great variability of the fungus reflects its heterokaryotic character. Cytological studies by Knox-Davies (11) showed that mature hyaline and pigmented hyphal cells of *Macrophomina* are uninucleate, but young growing hyphal cells and hyphal tip cells are usually multinucleate (12). Punithalingam (23), after further studies on nuclei of *M. phaseolina,* affirmed that during hyphal fusion heterokaryosis could occur after mitotic segregation and recombination. This may explain the occurrence of cultural types or physiological races reported.

New high oil varieties of sunflower have renewed interest in sunflower production in Italy. In recent years, sunflower has been widely cultivated not only in warm areas of the south and the center, but also in northern Italy (25) and in mountain areas (16) with colder climates. In all of these areas, CHR has been reported on sunflower (33,35).

In this paper, we report the results of studies to examine the response of 64 isolates of *M. phaseolina* to temperature, chlorate, and host inoculation. The aim was to investigate the adaptability of the fungus to different Italian climatic conditions under which sunflower is grown.

MATERIALS AND METHODS

Isolates from 64 different localities were chosen within four climatic areas: north (cold winter: −1/+2°C, wet area), mideast (mild winter: +4/+6°C, wet area), midwest (tepid winter: +6/+8°C, wet area), and south (tepid winter +6/+8°C, occasionally dry area), according to the Agro-climatic Atlas of Europe (32). Pathogenicity and cultural characteristics of these isolates were previously tested (15) and they were divided into three groups (highly virulent, virulent, and poorly virulent). They showed a wide variability unrelated to areas of origin. The pathogenicity of the north Italian population was not lower than that of populations from warmer areas.

Macrophomina phaseolina was isolated on potato-sucrose agar (PSA) from the lower portion of sunflower stems taken from 64 samples, out of 70 examined. Sample distribution was as follows: 16 from the north, 14 from the mideast, 14 from the midwest, and 20 from the south (15). The isolates were stored in silica gel (5).

Growth rate. Growth rate (GR) of 64 isolates was recorded at 15, 25, 30, 35, and 40°C. Culture disks, 4 mm diameter, cut from the edge of a 4-day-old PSA culture of each isolate, grown at 26°C, were transferred to the center of 9-cm petri dishes with 10 ml of PSA and incubated in the dark at the five different temperatures. Each treatment was replicated three times in a completely randomized design (9). The minor and major radii of the colonies were measured after 24 h, for 25, 30, and 35°C, and after 48 h for 15 and 40°C. These different incubation times were necessary because, after 24 h, at extreme temperatures the fungus often had not begun to grow, while at the other temperatures after 48 h it had often reached the edge of the plates. The final areas covered by the colonies were subjected to statistical analysis. Data were subjected to one way analysis of variance using the Statgraphics program, version 2.6 (Statistical Graphics

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Corp., Rockville, Md.), and to Scott-Knott cluster analysis (7). Average growth rates were subjected to one way analysis of variance for climatic area factor (mideast, south, north, and midwest) and to cluster analysis by the single linkage method using the Statgraphics program, version 2.6.

Sensitivity to potassium chlorate. *Macrophomina* isolates were examined for chlorate sensitivity, which reflects differences in nitrate assimilation (6,19), to identify any differences in metabolic adaptation to the different environments. Culture disks, 4 mm, cut from 6-day-old PSA cultures grown at 30°C, were transferred to 9-cm petri dishes with 10 ml of chlorate medium according to the method of Pearson et al. (19). Cultures were incubated in the dark at 30°C, then scored for growth 24 and 48 h after inoculation and compared with cultures grown on the same medium without chlorate.

Pathogenicity on eight plant species. The pathogenicity of 24 isolates from the four climatic areas and from the three groups of virulence (15) (highly virulent, virulent, and poorly virulent on sunflower), were tested on eight plant species: soybean (*Glycine max* (L.) Merr.) cv. Azzurra, maize (*Zea mays* L.) cv. Ivana, sorghum (*Sorghum vulgare* L.) cv. NK 121, sunflower (*Helianthus annuus* L.) cv. Francasol, safflower (*Carthamus tinctorius* L.) cv. Elena, sugar beet (*Beta vulgaris* L.) cv. Cermo, kenaf (*Hibiscus cannabinus* L.) cv. BG 2436, and melon (*Cucumis melo* L.) cv. Retato degli Ortolani. Pathogenicity testing was carried out at the seedling stage. Seeds of commercial varieties were sterilized with 2% sodium hypochlorite for 4 min and rinsed twice in sterile tap water. Seeds were placed on 6-day-old colonies of each *Macrophomina* isolate grown on PSA in 9-cm plates at 30°C in the dark. Each treatment of six seeds, arranged in two plates, was replicated three times in a completely randomized block design (9). Plates were incubated at 26°C in the dark and 2 ml of sterile tap water was added to each plate to promote seed germination. Pathogenicity was recorded 4 and 8 days after seed inoculation using the following severity assessment key: 0 = healthy seed; 1 = discoloration of a portion of the seedling in contact with the mycelium; 2 = seed teguments invaded by mycelium and sclerotia but healthy seedling; 3 = seed teguments free from the fungus, but seedling infected; 4 = seed tegument and seedling infected; 5 = seed infected and not germinated. The disease index was calculated by multiplying the number of seeds by the degree of disease severity (i.e., six seeds with disease severity of 4 = 24). The data were subjected to two way analysis of variance and a least significant difference test using the Statgraphics program, version 2.6. The experiment was repeated twice with the same results.

RESULTS

GR. A temperature of 30°C was optimal for 62 isolates and 35°C for two isolates. The mean area of the colonies grown at 25, 30, and 35°C was 2.6, 8.0, and 5.4 cm^2, respectively, after 24 h; the mean of colonies grown at 15 and 40°C was 0.7 and 1.7 cm^2, respectively, after 48 h. Analysis of variance of GR at five different temperatures was always significant ($P < 0.01$), but the maximum variation between isolates occurred at 15 and 40°C with an F ratio of 19.7 and 23.2, respectively, while for GR at 25, 30, and 35°C the F ratio was lower: 2.5, 7.5, and 6.4, respectively. Cluster analysis of GR data showed that isolates could be better differentiated at 15 and 40°C than at the optimal temperatures. In the 15°C GR test, only the isolates from the north had values in clusters A and B and highest frequency in C, while GR values of isolates from warmer areas predominated in clusters E and F (Table 1, Fig. 1A). All the isolates fell within two clusters for the 25°C GR test and within three clusters for the 30°C GR test (Fig. 1B, C). In the 35°C GR test only some isolates from the mideast and south had values in cluster A, but the difference between GR values was very low and the majority of the means fell in clusters B and C (Table 1, Fig. 1D). In the 25, 30, and 35°C GR tests, the mean separation did not distinguish any group according to climatic area. At 40°C isolates from the midwest had the best GR (clusters A, B, and C) and the lowest frequency of isolates in clusters E and F (Table 1, Fig. 1E). Only isolates from the north had high GR values (clusters B and C), while isolates from the mideast and south had low GR values (clusters E and F).

One way analysis of variance for the climatic area factor of 15°C GR was significant ($P < 0.01$). The northern area was significantly different from the other areas. Differences between climatic areas were also significant ($P < 0.01$) for GR at 25 and 30°C, but less than for 15°C (Table 2). At 35°C, there was no significant difference, while the difference was significant ($P < 0.01$) at 40°C, at which isolates from the midwest had higher GR values (Table 2).

Cluster analysis of the limit temperatures and climatic areas in a three dimensional plot gave an indicative separation of the means (Fig. 2): the mideastern and southern areas fell within the same cluster even if the southern isolates had lower values of 15°C GR and higher values of 40°C GR. The northern and midwestern isolates were clearly distinct: the north tended to high values of 15°C GR; the midwest concentrated around a low value of 15°C GR (Fig. 2). The same cluster analysis of GR with other temperature combinations always gave a wide data spread around the mean GR.

Isolates from different climatic areas maintained their thermophilic character with optimum temperatures of 30 and 35°C. Isolates from the north were able to grow better at low temperature than isolates from other areas where the pathogen has always been present. Those isolates from areas with low limit temperature adapted well to extreme conditions; their GR at 40°C was better than that of isolates from mideastern and southern Italy and they maintained the thermophilic character and their pathogenicity on sunflower did not differ from isolates from other areas (15). Isolates from the mideast and south, which have good climatic conditions for CHR, tended to grow better at optimum temperatures (30 and 35°C). GR differences between four of the *Macrophomina* populations at optimum temperatures were minimum and the mean separation with Scott-Knott analysis of GR values did not distinguish any group according to climatic area (Fig. 1C, D). Isolates from the midwest had the best growth rate at 40°C, but they had the worst GR value at 15°C; they only adapted well to the warmer temperatures.

Sensitivity to potassium chlorate. Four colony chlorate phenotypes were observed: 1 = dense growth like that obtained without potassium chlorate (only 1 isolate); 2 = normal growth of colony, but less dense than growth without potassium chlorate (30 isolates); 3 = feathery spreading growth (31 isolates); 4 = restricted growth (2 isolates). Almost all Italian isolates were chlorate tolerant (95%), but chlorate tolerance was variable: 47% produced a colony less dense than the control and 48% showed a feathery phenotype. Chlorate sensitivity was not related to climatic areas.

Table 1. Intervals of growth rate (GR) values (cm^2) of 64 isolates of *Macrophomina phaseolina* at five temperatures subjected to mean separation using the Scott-Knott cluster analysis method[y]

	Temperature				
Clusters	**15°C*[z]**	**25°C**[z]**	**30°C**[z]**	**35°C**[z]**	**40°C*[z]**
A	5.8	2.8 to 4.1	10.1 to 11.5	7.3 to 8.5	4.4
B	3.9 to 4.5	1.3 to 2.7	8.3 to 9.7	5.5 to 6.9	2.8 to 3.1
C	3.0 to 3.6		6.8 to 8.2	4.1 to 5.4	1.8 to 2.1
D	2.3 to 2.9		4.8 to 6.6	3.2 to 4.0	1.3 to 1.7
E	1.3 to 2.2			1.9	0.5 to 1.2
F	0.3 to 1.2				0 to 0.4

[y] Values are the mean of three replications.
[z] * = GR recorded after 48 h; ** = GR recorded after 24 h.

Pathogenicity test. The pathogenicity of 24 isolates of *Macrophomina* from sunflower was significantly different ($P < 0.01$) on eight plant species. None of the isolates were pathogenic on maize and all were pathogenic on other species. Isolates were highly virulent on soybean, virulent on sunflower, safflower, sorghum, and melon, and mildly virulent on kenaf and sugar beet (Table 3). The virulence of isolates on each host species did not vary widely with the exception of kenaf (standard error 1.1), in which pathogenicity varied from 0 (mean value of three isolates) to 27 (mean pathogenicity of one isolate).

DISCUSSION

The environmental conditions of the mideastern and southern areas of Italy, on the Adriatic coast, are similar but differ from those of the midwestern, on the Tyrrhenian coast. It is probable that environmental conditions and cropping systems in the midwest have encouraged *Macrophomina* isolates to adapt to the higher temperature better than in other warm areas. On the basis of this study, we can affirm that combined GR values at 15 and 40°C can be indicative of climatic areas of origin and that the fungus has adapted to these climatic conditions, which are far from the best, especially the colder conditions. This means that CHR can potentially occur in colder areas of Italy where, in dry summers, the pathogen has a high inci-

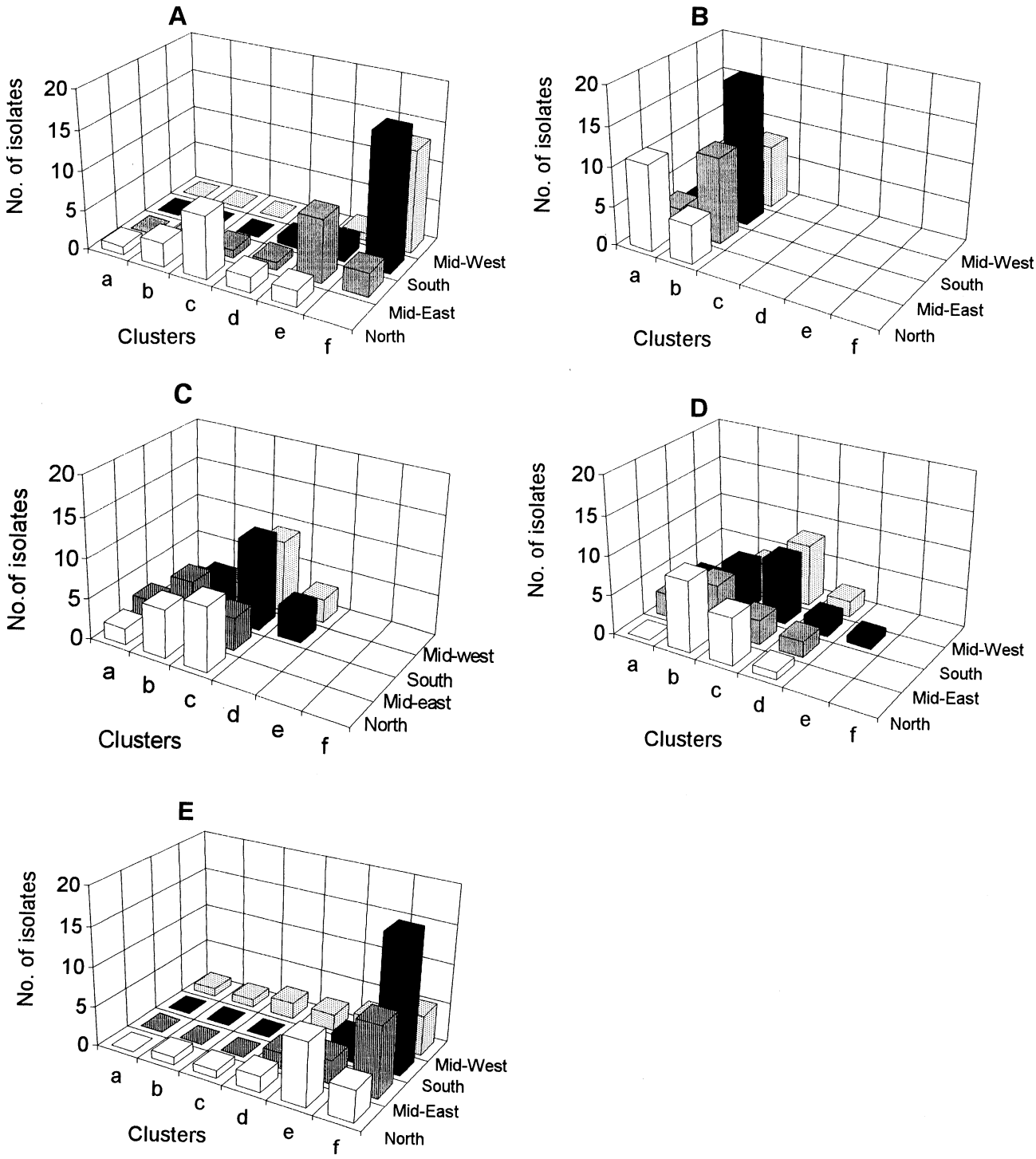

Fig. 1. Frequency of 64 isolates of *Macrophomina* from four Italian sunflower cultivation areas, in growth rate (GR) clusters at five different temperatures obtained using mean separation by Scott-Knott cluster analysis. **(A)** 15°C. **(B)** 25°C. **(C)** 30°C. **(D)** 35°C. **(E)** 40°C.

dence on the crop while in wet summers *Sclerotinia sclerotiorum* occurs on the stem (17,34).

Only 3% of isolates were chlorate resistant and 1% were chlorate sensitive. Pearson et al. (20), comparing the chlorate sensitivity of *M. phaseolina* isolates from soybean, maize, sunflower, sorghum, and other hosts, found that most of the sunflower and soybean isolates were either chlorate tolerant or chlorate sensitive whereas maize and sorghum isolates were predominantly chlorate resistant. After further studies on nitrogen source utilization by chlorate-resistant and chlorate-sensitive *Macrophomina* isolates, Pearson et al. found that isolates from soybean had a high level of nitrate reductase while those from maize had a low basal level (21). This is due to the different nitrogen composition in the xylem of maize and soybean, which varies according to the species (18). Furthermore, Pearson et al. found that under extremely high stress levels host preference of chlorate-tolerant isolates can be overcome because their specialization is not as high as that of chlorate resistant and chlorate sensitive isolates (20). Chlorate sensitivity of our sunflower isolates did not seem to be related to origin: the variability between chlorate tolerant isolates could be due to the heterokaryotic character of the fungus.

Pathogenicity tests on eight plant species confirmed the wide variability among isolates and confirmed, in part, the chlorate sensitivity of the isolates: all sunflower isolates were pathogenic on soybean and not on maize. The only chlorate-resistant isolate inoculated on seedlings of eight plant species did not differ from chlorate-tolerant isolates. The pathogenicity of two chlorate-sensitive isolates was not tested on different species but, like all the sunflower isolates, they were pathogenic even if mildly so. At present, the specialization of isolates was tested on seedlings, while their specialization to the host also seems to be related to stem nitrogen composition and promoted under water-stress conditions (21); therefore, pathogenicity on seedlings is probably not always related to chlorate sensitivity. Furthermore, the variation in host sensitivity to *Macrophomina* can depend upon the growth stage (28). This might explain the high level of pathogenicity of sunflower isolates on sorghum that have a chlorate-resistant *Macrophomina* population (20), but it could also be due merely to a sorghum preference for Italian chlorate-tolerant populations. Sunflower isolates, besides soybean and sorghum, were also pathogenic on safflower and melon whereas they were mildly pathogenic on sugar beet and kenaf. Sorghum, sugar beet, and soybean are often used in rotation with sunflower in Italy, while kenaf and safflower are two new alternative crops to sunflower in dry and warmer areas. The sensitivity of those species to *M. phaseolina* should be considered when choosing the cropping sequence, as this might influence the level of *M. phaseolina* in the soil (29). The pathogenicity of sunflower isolates on melon showed that the fungus could be widespread on vegetable crops; e.g., melon and strawberries have been reported as hosts in other countries (13, 14).

This study on variability of sunflower isolates shows that *Macrophomina* is a widespread sunflower pathogen and, probably because of its heterokaryotic character, has been able to adapt to the low temperatures of new sunflower cultivation ar-

Table 2. Growth rate (GR) difference of 64 isolates of *Macrophomina phaseolina* from four climatic areas. Values are expressed as cm^2 of colonies recorded after 24 h for the 25, 30, and 35°C GR tests and after 48 h for the 15 and 40°C GR tests

GR tests	Climatic areas	No. of isolates	Average (cm^2)
15°C	Midwest	14	0.80 a
	South	20	0.93 a
	Mideast	14	1.85 b
	North	16	3.48 c
25°C	South	20	2.24 a
	Mideast	14	2.30 a
	Midwest	14	2.90 b
	North	16	3.08 b
30°C	Midwest	14	7.26 a
	South	20	7.38 a
	North	14	8.54 b
	Mideast	14	9.20 b
35°C	Midwest	14	4.83 a
	South	20	5.26 a
	North	16	5.60 a
	Mideast	14	5.81 a
40°C	South	20	0.30 a
	Mideast	14	0.49 a
	North	16	0.99 bc
	Midwest	14	1.37 c

[z] Values are the means for isolates from each climatic area (value of each isolate is mean of three replications). Mean values followed by the same letter do not differ at $P < 0.05$ according to a least significant difference test.

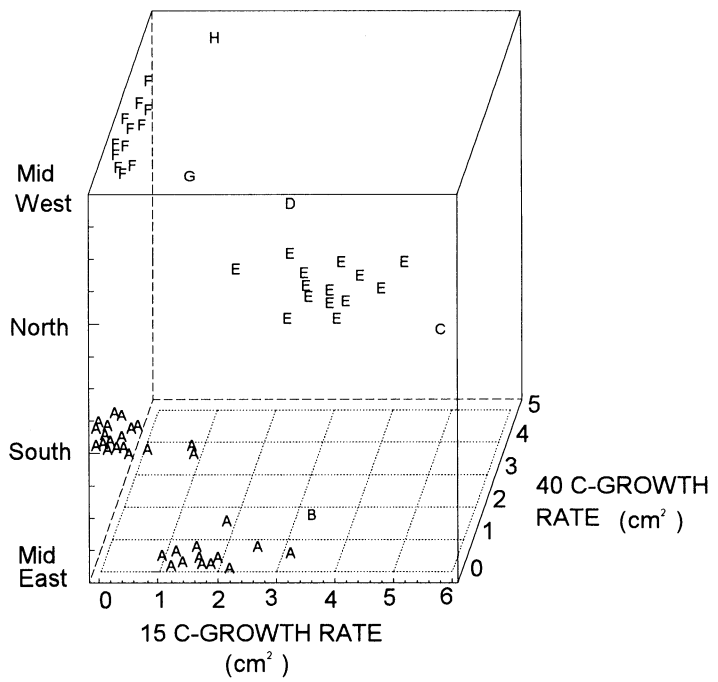

Fig. 2. Results in three-dimensional plot of clustering using the single linkage method. X = growth rate (GR) at 15°C, Y= GR at 40°C, Z= climatic areas.

Table 3. Pathogenicity of 24 isolates of *Macrophomina phaseolina* on eight plant species

Species	Cultivars	Average[z]	Standard error
Zea mays	Ivana	0 a	0.0
Beta vulgaris	Cermo	7.4 b	0.6
Hibiscus cannabinus	BG 2436	10.5 c	1.1
Cucumis melo	Retato degli Ortolani	22.1 d	0.5
Sorghum vulgare	NK 121	22.3 d	0.3
Carthamus tinctorius	Elena	23.5 d	0.5
Helianthus annuus	Francasol	23.6 d	0.2
Glycine max	Azzurra	28.6 e	0.2

[z] Data are the mean of three replications of each species. Mean values followed by the same letter do not differ at $P < 0.01$ according to a least significant difference test. Severity was recorded according to a 0 to 5 scale. Severity multiplied by number of diseased seeds = pathogenicity.

eas in northern Italy characterized by a continental climate. Moreover, the pathogenicity of isolates on seedlings of eight host species shows that *Macrophomina* from sunflower may be a potential pathogen on other crops, especially under water-stressed conditions.

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