Eur. J. For. Path. 25 (1995) 232–239

ISSN 0300–1237

Istituto di Patologia vegetale, Università di Padova, Italy

Vegetative compatibility and conversion to hypovirulence among Italian isolates of *Cryphonectria parasitica*

By R. CAUSIN, G. FRIGIMELICA, L. MONTECCHIO and S. MUTTO ACCORDI

Summary

A total of 850 virulent isolates of *Cryphonectria parasitica* were isolated from natural cankers on European chestnut trees growing in various Italian regions. Vegetative-compatibility (v-c) group membership was tested using the merge-barrage method. In all, 19 vegetative-compatibility groups were found, and, for each of these, a representative isolate (v-c tester) was identified; 49% of the isolates were compatible with the tester of only one v-c group, whereas 51% were compatible with the testers of more than one v-c group; 3% of the isolates showed compatibility with seven v-c groups. The coincidence between vegetative incompatibility and failure of conversion occurred in only a few cases; 42% of the converts were stable with respect to their morphological characteristics and presence of cytoplasmic ds-RNA. The results obtained in Italy suggest that, for an efficient biocontrol programme, it is preferable to evaluate the tendency of a population to be converted rather than evaluating the v-c group membership.

1 Introduction

A decrease in the epidemic of European chestnut blight coincided with the spread of hypovirulent isolates of *Cryphonectria parasitica* (Murr.) Barr. (GRENTE 1965). These isolates contained viral double-stranded RNAs (ds-RNAs; CHOI and NUSS 1992) which, when transmitted by hyphal anastomoses to mycelia of 'normal' pathogenic isolates, modify their morphology and attenuate their pathogenicity. The distribution of these hypovirulent isolates in Italy is, however, not uniform (LUISI 1984; GOBBI and LOCCI 1989; PENNISI et al. 1991, 1992; MAGRO et al. 1992). In some areas, hypovirulent isolates seem to predominate, while, in others, the virulent form prevails. The prevalence of hypovirulent isolates may be related to the different numbers of vegetative compatibility groups within local populations of *C. parasitica* (GOBBI and LOCCI 1989; PENNISI et al. 1991, 1992). Some authors have reported that the vegetative incompatibility may limit or inhibit the transmission of the ds-RNA (ANAGNOSTAKIS 1977; NEWHOUSE and MACDONALD 1991). A complete understanding of the vegetative-compatibility status of a population of *C. parasitica* may be fundamental to the establishment of an efficacious plan for biological control of the disease. Studies made in Italy to date have only examined regional situations (LUISI 1984; GOBBI and LOCCI 1989; PENNISI et al. 1991, 1992; MAGRO et al. 1992). In this study, vegetative-compatibility groups that exist throughout Italy were examined. Because the transmission of hypovirulence is possible even among vegetatively incompatible individuals (ANAGNOSTAKIS and WAGGONER 1981; ANAGNOSTAKIS 1983; KUHLMAN and BHATTACHARYYA 1984), the relationship between vegetative compatibility and conversion to hypovirulence of a sample of Italian virulent isolates was determined.

2 Materials and methods

2.1 Sampling of the isolates and their characterization

Between 1989 and 1994, 850 virulent isolates were collected in Veneto, Trentino-Alto Adige, Friuli-Venezia Giulia, Lombardia, Piemonte, Liguria, Toscana, Emilia Romagna, Lazio,

Campania, Calabria, Basilicata, and Sicilia. The mycelia were isolated and cultured according to methods described by ANAGNOSTAKIS et al. (1986). They were classified as virulent (V) or hypovirulent (H) on the basis of their morphological characteristics (GRENTE 1981; ELLISTON 1985).

2.2 Vegetative compatibility (v-c) groups

A sample of 50 virulent isolates was chosen at random for tester selection. These isolates, obtained from Veneto, Toscana and Lazio, were then paired in all possible combinations using the method described by KUHLMAN (1983). The number of v-c groups and the isolates representative of each v-c group were determined as described previously (KUHLMAN et al. 1984; GARBELOTTO 1990; GARBELOTTO et al. 1992). Ten monoconidial isolates were prepared from each representative isolate (GARBELOTTO et al. 1992). For each v-c group, one of these monoconidial isolates was chosen at random. If the selected monoconidial isolate was fully compatible with the isolate from which it originated, it was considered a tester and used for pairings with all other monoconidial isolates. When a monoconidial isolate showed no incompatibility with monoconidial isolates of other v-c groups, it was excluded and the v-c tester determination was repeated using another monoconidial isolate. Each V isolate was then paired with the v-c testers (KUHLMAN and BHATTACHARYYA 1984). Pairs of isolates that merged were placed in the same v-c group, whereas those that formed lines of pycnidia (barrage) were placed in different v-c groups (KUHLMAN 1983).

Merging with the formation of a barrage later on was classified as a no-merge response. Weak barraging (ANAGNOSTAKIS 1983) with the formation of permanent merging areas was considered a merge response (GARBELOTTO et al. 1992). The isolates incompatible with all the v-c testers were placed in a separate group. As soon as this group contained 10 isolates, they were paired with each other as described above to identify new v-c groups and to identify their testers.

2.3 Conversion

The v-c testers and two randomly selected isolates from each of the 12 most frequent v-c groups were paired (KUHLMAN et al. 1984) with four monoconidial H isolates randomly chosen among those that demonstrated incompatibility among themselves as well as with the majority of the v-c testers. They originated from four different provinces (H1: province of Verona, Veneto; H2: province of Cosenza, Calabria; H3: province of Viterbo, Lazio; H4: province of Padova, Veneto).

Four replicate pairings were made to check rate of conversion. The v-c of the four isolates was evaluated using the isogenic V isolates obtained as previously described (GARBELOTTO et al. 1992); 30% of the transformed isolates, their corresponding V receptors, and their transformers, were subjected to ds-RNA analysis (GARBELOTTO et al. 1992) to confirm that the ds-RNA transfer had occurred.

2.4 Stability of the conversion

From the converted sectors of 16 randomly selected V isolates, mycelial plugs (4-mm diameter) were sampled and subsequently grown on PDA with a 16 h/day photoperiod (fluorescent light) at 25 °C for 7 days. From these cultures, three successive subcultures were obtained weekly and inoculated under the same conditions to test the persistence of the H morphology. The stable converts were grown on PDA for 5 days in the dark at 25 °C, after which they were inoculated (MONTECCHIO et al. 1994) on cut segments of European chestnut (*Castanea sativa* Mill.) stems (length 30 cm, diameter 3 cm) whose bases were

immersed in 10 cm of water (16 h/day daylight; 20°C). Twenty days after inoculation, the mycelia were isolated again from the margin of the necrotic area. If the mycelia maintained their H morphology, they were tested for the presence of ds-RNAs and for their ability to convert the initial V isolate.

3 Results

3.1 Vegetative-compatibility (v-c) groups

A total of 19 v-c groups were detected, most of which contained isolates from various regions. Of the 850 isolates tested, 416 were compatible only with the tester of its own group and were distributed among 19 v-c groups (Fig. 1). The remaining 434 isolates showed the same compatibility merge response with testers of several v-c groups (Fig. 2); 51% of these isolates were compatible with at least two groups, and a limited number (3%) demonstrated compatibility with seven different groups. In 92% of all confrontations with the testers, the presence or absence of a clear barrage zone was evident. Only in 8% of pairings did the method described by GARBELOTTO et al. (1992) have to be used to determine whether there was a merge response or not.

3.2 Conversion

Although the four H isolates were incompatible with each other, some of them were compatible with the same v-c tester (Table 1). H1 and H4 were compatible with T1 and T2, and H2 and H3 were compatible with T12. None of the four H isolates examined was able to convert T11, but V11a was converted by H3 and H4, and V11b was converted by all four H isolates. In all the other cases, the isolates were converted by at least one of the four H isolates tested.

All the transformed isolates tested contained ds-RNA similar to that present in the respective H transformers. Prior to transformation, the V isolates were devoid of ds-RNA.

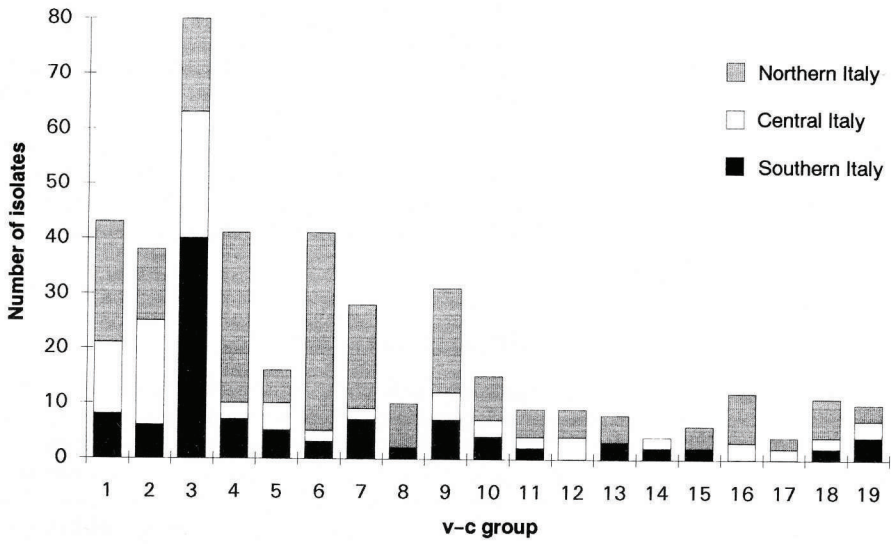

Fig. 1. Distribution of isolates from various regions of Italy among v-c groups

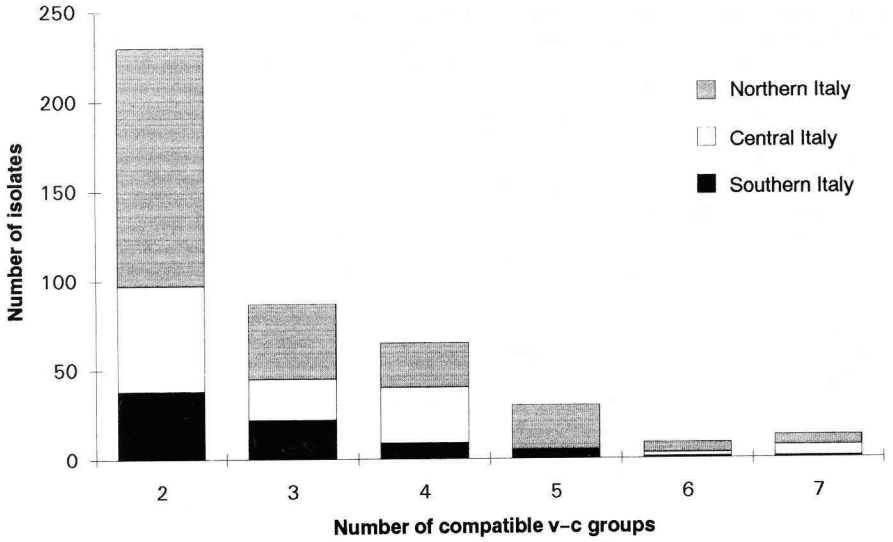

Fig. 2. Number of isolates compatible with testers of more than one v-c group

3.3 Stability of conversion

The trials demonstrated that 41.66% of the converts were stable *in vitro*, maintained the ds-RNA and a typical H morphology, as well as the capability to convert the initial V isolate *in vivo*.

4 Discussion

This study has outlined the great degree of homogeneity in the sample examined, attested by the low number of v-c groups identified and by the remarkable number of isolates that can be placed into many v-c groups (KUHLMAN et al. 1984; MILGROOM et al. 1991). Other authors have found multiple merge v-c groups in studies carried out in other countries (KUHLMAN and BHATTACHARYYA 1984; KUHLMAN et al. 1984). The 19 different v-c groups identified consisted of isolates from everywhere in Italy. These data are similar to those in other Italian studies (PENNISI et al. 1991, 1992), but differ significantly from those reported for North America (ANAGNOSTAKIS et al. 1986). The difference in the frequency of v-c groups between Italy and North America could be attributed to the frequency of occurrence of the sexual form in the two countries. In North America, it has been hypothesized that ascospores are the major source of inoculum (MACDONALD and DOUBLE 1978; ANAGNOSTAKIS 1987), whereas, in Italy, until 1992, the production of perithecia was a rare event (TURCHETTI 1987; S. MUTTO ACCORDI pers. obs.; A. ZAMBONELLI pers. comm.). Such a situation also parallels the events encountered in France, where sexual fructifications were rare (GRENTE 1981). Only recently have perithecia been observed in some Italian regions, but only at very low frequency (S. MUTTO ACCORDI pers. obs.; T. TURCHETTI and A. ZAMBONELLI pers. comm.). In some areas of Italy, high degrees of both v-c homogeneity and heterogeneity have been reported (LUISI 1984; GOBBI and LOCCI 1989; PENNISI et al. 1991, 1992), but those results refer to circumscribed areas and cannot be extended to the whole Italian territory. Furthermore, the results obtained indicate that the formation of v-c groups using v-c testers can lead to erroneous evaluations, in accordance with other findings (KUHLMAN and BHATTACHARYYA 1984). Some isolates incompatible

Table 1. **Percentage of conversion of virulent isolates by the hypovirulent isolates H1-H4 (number of converted sectors × 100/total number of sectors paired) and vegetative compatibility between testers and hypovirulent isolates. T1–T12 = tester isolates of 12 major v-c groups; V1a, V1b to V12a, V12b = virulent isolates belonging to the 12 major v-c groups; + = compatible; − = incompatible**

Virulent isolates	Hypovirulent isolates							
	Conversion (%)				Compatibility			
	H1	H2	H3	H4	H1	H2	H3	H4
T1	100	100	100	100	+	−	−	+
V1a	100	100	100	100				
V1b	100	100	100	100				
T2	100	100	100	100	+	−	−	+
V2a	100	100	100	100				
V2b	100	75	62.5	62.5				
T3	100	100	37.5	100	−	+	−	−
V3a	100	50	75	100				
V3b	100	100	100	75				
T4	25	100	75	100	−	−	−	−
V4a	25	0	25	0				
V4b	25	12.5	50	50				
T5	100	75	100	75	−	−	−	−
V5a	100	100	100	100				
V5b	100	37.5	75	100				
T6	100	0	0	12.5	−	−	−	−
V6a	0	37.5	0	25				
V6b	50	75	50	75				
T7	12.5	25	37.5	100	−	−	−	−
V7a	75	25	37.5	75				
V7b	50	0	25	100				
T8	0	100	50	100	−	−	−	−
V8a	75	50	25	12.5				
V8b	100	100	100	0				
T9	100	100	100	100	−	−	+	−
V9a	100	75	100	100				
V9b	100	100	75	0				
T10	0	0	100	100	−	−	−	−
V10a	100	100	100	12.5				
V10b	100	100	25	100				
T11	0	0	0	0	−	−	−	−
V11a	0	0	62.5	50				
V11b	100	75	100	100				
T12	0	100	100	62.5	−	+	+	−
V12a	100	100	62.5	100				
V12b	100	100	100	100				

with each other were compatible with the same v-c tester and were therefore assigned to the same v-c group. The H isolates converted most of the V isolates with which they were incompatible. This confirms that there is not always a direct relationship between absence of conversion and incompatibility, as determined by the merge–barrage system. This result is in accordance with other authors' findings (ANAGNOSTAKIS 1983; ANAGNOSTAKIS and DAY 1979; ANAGNOSTAKIS and WAGGONER 1981; KUHLMAN and BHATTACHARYYA 1984). The merge–barrage technique is based on a macroscopic evaluation of the interaction between colonies and it does not allow for an early evaluation of the events that take place during the first phase of contact between the hyphae of the opposed mycelia. In this early

phase, it is possible that temporary anastomoses may form and degenerate, giving rise, more or less rapidly, to the formation of a barrage. What cannot be excluded is the passage of the ds-RNA prior to degeneration (MARTIN and VAN ALFEN 1991). The results of this study demonstrate that the passage of the ds-RNA can take place when a barrage is formed and following conversion often remain stable even after inoculation into host tissue. This may imply that a network or relationship exists among conversion and vegetative compatibility, but no pattern is clear because v-c is controlled by at least five genes, and relationships among v-c groups are known only for a few selected groups from lab crosses (GRENTE 1981; ANAGNOSTAKIS 1977, 1988; HUBER and FULBRIGHT 1994).

KUHLMAN'S work on conversion has led to a concept of hypovirulence-conversion compatibility (KUHLMAN and BHATTACHARYYA 1984; KUHLMAN et al. 1984). The results obtained in Italy in this study validate KUHLMAN'S findings and suggest that, for an efficient biocontrol programme, it is preferable to evaluate the aptitude toward conversion of the population rather than its v-c. This latter parameter does not always represent the true tendency of a population to be converted.

Acknowledgements

The authors thank Professors M. BISIACH, G. MAGNANO DI SAN LIO, P. MAGRO and N. LUISI, who sent some of the isolates of *C. parasitica*, as well as Prof. W. L. MACDONALD for the review of the manuscript.

Résumé

Compatibilité végétative et conversion à l'hypovirulence chez des isolats italiens de Cryphonectria parasitica

Huit cent cinquante isolats de *Cryphonectria parasitica* ont été isolés de chancres naturels de châtaigniers dans diverses régions d'Italie. L'appartenance aux groupes de compatibilité végétative (v-c) a été testée par confrontation. Dix neuf groupes de compatibilité ont été trouvés et pour chacun d'eux, un isolat représentatif (testeur v-c) a été désigné; 49% des isolats étaient compatibles avec le testeur d'un groupe v-c seulement, alors que 51% étaient compatibles avec les testeurs de plus d'un groupe v-c; 3% des isolats montraient compatibilité avec sept groupes v-c. La coïncidence entre l'incompatibilité végétative et l'absence de conversion avait lieu dans seulement quelques cas; 42% des isolats convertis étaient stables pour les caractères morphologiques et pour la présence de ds-RNA cytoplasmique. Ces résultats obtenus en Italie suggèrent que pour un programme de contrôle biologique efficace il est préférable d'évaluer la tendance d'une population à être convertie plutôt que l'appartenance aux groupes v-c.

Zusammenfassung

Vegetative Kompatibilität und Übertragung der Hypovirulenz bei Cryphonectria parasitica Isolaten aus Italien

Insgesamt 850 virulente Stämme von *Cryphonectria parasitica* wurden von natürlichen Krebsen an Edelkastantien in den verschiedensten Regionen Italiens isoliert. Die Zugehörigkeit zu den verschiedenen Kompatibilitätsgruppen (v-c) wurde durch paarweises Kreuzen der Isolate auf einem Agarmedium ermittelt. Verschmolzen zwei Isolate ohne sichtliche Ausbildung einer Barrierezone, wurden sie als kompatibel, bildeten sie dagegen im Berührungsbereich eine Barrierezone aus, wurden sie als inkompatibel eingestuft. Es wurden 19 Kompatibilitätsgruppen gefunden, und für jede von diesen wurde ein Isolat (v-c Tester) als Repräsentant ausgewählt; 49% der Isolate waren nur mit dem Tester einer einzigen v-c Gruppe, die restlichen 51% mit den Testern mehrerer v-c Gruppen kompatibel; 3% der Isolate zeigten Kompatibilität mit sieben v-c Gruppen. Nur wenige Isolate waren miteinander kompatibel und zugleich nicht zur Übertragung der Hypovirulenz fähig; 42% der konvertierten Stämme waren bezüglich der Kulturmorphologie und dem Vorhandensein zytoplasmatischer ds-RNA stabil. Diese für italienische Isolate erhaltenen Resultate bestätigen die Befunde von Kuhlman und lassen

vermuten, daß es für ein effizientes Programm zur biologischen Bekämpfung besser ist, die Übertragbarkeit der Hypovirulenz in einer *C. parasitica*-Population zu untersuchen als deren Zugehörigkeit zu den v-c Gruppen.

References

ANAGNOSTAKIS, S. L., 1977: Vegetative incompatibility in *Endothia parasitica*. Experimental Mycol. **1,** 306–316.

–, 1983: Conversion to curative morphology in *Endothia parasitica* and its restriction by vegetative compatibility. Mycologia **75,** 777–780.

–, 1987: Chestnut blight: the classical problem of an introduced pathogen. Mycologia **79,** 23–37.

–, 1988: *Cryphonectria parasitica* cause of chestnut blight. Adv. Plant Pathol. **6,** 123–136.

–; DAY, P. R., 1979: Hypovirulence conversion in *Endothia parasitica*. Phytopathology **69,** 1226–1229.

–; WAGGONER, P. E., 1981: Hypovirulence, vegetative compatibility, and the growth of cankers of chestnut blight. Phytopathology **71,** 1198–1202.

–; HAU, B.; KRANZ, J., 1986: Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. Pl. Dis. **70,** 536–538.

CHOI, G. H.; NUSS, D. L., 1992: Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science **257,** 800–803.

ELLISTON, J. E., 1985: Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology **75,** 151–158.

GARBELOTTO, M., 1990: Vegetative compatibility in *Cryphonectria parasitica*. Tesi di laurea, Università degli Studi di Padova, Italy.

–; FRIGIMELICA, G.; MUTTO ACCORDI S., 1992: Vegetative compatibility and conversion to hypovirulence among isolates of *Cryphonectria parasitica* from northern Italy. Eur. J. For. Path. **22,** 337–348.

GOBBI, E.; LOCCI, R., 1989: Aspetti micologici e fitopatologici di *Cryphonectria parasitica*. Mic. Ital. **3,** 31–36.

GRENTE, J., 1965: Les formes hypovirulentes d'*Endothia parasitiaca* et les espoirs de lutte contre le chancre du chataignier. Academie d'Agricolture de France **51,** 1033–1037.

–, 1981: Les variants hypovirulents de l'*Endothia parasitica* et la lutte biologique contra le chancre du chataignier. PhD thesis, Université de Bretagne Occidentale, Brest, France.

HUBER, D. H.; FULBRIGHT, D. W., 1994: Preliminary investigations on the effect of individual vic genes upon the transmission of dsRNA in *Cryphonectria parasitica*. In: Proc. Int. Chestnut Conf. Ed. by DOUBLE, M. L.; MACDONALD, W. L. Morgantown: West Virginia University Press. pp. 15–19.

KUHLMAN, E. G., 1983: Effects of hypovirulence in *Cryphonectria parasitica* and of secondary blight infections on dieback of American chestnut trees. Phytopathology **73,** 1030–1034.

–; BHATTACHARYYA, H., 1984: Vegetative compatibility and hypovirulence conversion among naturally occurring isolates of *Cryphonectria parasitica*. Phytopathology **74,** 659–664.

–; –; NASH, B. L.; DOUBLE, M. L.; MACDONALD, W. L., 1984: Identifying hypovirulent isolates of *Cryphonectria parasitica* with broad conversion capacity. Phytopathology **74,** 676–682.

LUISI, N., 1984: Situazione fitopatologia del cancro corticale del Castagno e prospettive di risanamento. In: Proc. 'Caratteristiche agronomiche, economiche e fitopatologiche del castagno del Vulture'. Ed. by Comunità Montana Vulture. Melfi, Italy. pp. 3–21.

MACDONALD, W. L.; DOUBLE, M. L., 1978: Frequency of vegetative compatibility types of *Endothia parasitica* in two areas of West Virgina. In: Proc. American Chestnut Symp. Ed. by MACDONALD, W. L.; CECH, F. C.; LUCHOK, J.; SMITH, C. Morgantown: West Virgina University Press. pp. 103–105.

MAGRO, P.; BISIACH, M.; MUTTO ACCORDI, S.; TURCHETTI, T., 1992: Il cancro del castagno in Italia e le prospettive di impiego degli isolati ipovirulenti di *Cryphonectria (Endothia) parasitica* nel controllo della malattia. In: Proc. 'Piante forestali — Avversità biotiche e prospettive di controllo biologico e integrato'. Ed. by COVASSI, M. Firenze, Italy. pp. 61–77.

MARTIN, R. M.; VAN ALFEN, N. K., 1991: The movement of Viral-Like RNA between colonies of *Cryphonectria parasitica*. Molecular Plant-Microbe Interactions **4,** 507–511.

MILGROOM, M. G.; MACDONALD, W. L.; DOUBLE, M. L., 1991: Spatial pattern analysis of vegetative compatibility groups in the chestnut blight fungus, *Cryphonectria parasitica*. Can. J. Bot. **69,** 1407–1413.

MONTECCHIO, L.; CAUSIN, R.; MUTTO ACCORDI, S., 1994: Variazione nel tempo della recettività a *Ceratocystis fimbriata* f. sp. *platani* di tessuti di platano feriti. Petria **4,** 47–56.

NEWHOUSE, J. R.; MACDONALD, W. L., 1991: The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. Can. J. Bot. **69,** 602–614.

PENNISI, A. M.; GRANATA, G.; SIDOTI, A., 1991: Diffusione ed aspetti micologi di *Cryphonectria parasitica* in Calabria e Sicilia. Mic. Ital. **3**, 21–26.

–; MAGNANO DI SAN LIO, G.; GRASSO, S., 1992: Compatibilità vegetativa di isolati di *Cryphonectria parasitica* (Murr.) Barr ottenuti da castagneti in Calabria. Petria **2**, 1–10.

TURCHETTI, T., 1987: Prospettive di lotta biologica in patologia forestale. In: Proc. 'Convegno sulle avversità del bosco e delle specie arboree da legno'. Ed. by Accademia Nazionale Italiana di Entomologia. Accademia Italiana di Scienze Forestali Firenze, Italy. pp. 265–276.

Authors' addresses: R. CAUSIN, L. MONTECCHIO and S. MUTTO ACCORDI: Istituto di Patologia vegetale, Università di Padova, Via Gradenigo 6, I-35131 Padova, Italy; G. FRIGIMELICA, Via Tisoi 6, I-32020 Belluno, Italy

Received: 12.9.94; accepted: 23.5.95