International Journal of Horticultural Science 2006, 12 (1): 63–67
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Genetic variation in the rose pathogen *Marssonina rosae* estimated by RAPD

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Summary Blackspot, caused by the fungus *Marssonina rosae* (*Diplocarpon rosae*), is one of the most devastating and widespread diseases in garden roses, and it has as yet not been fully characterized in molecular terms. In this initial study we used RAPD analysis to investigate the genetic diversity among and within a few geographically diverse groups of single-spore isolates of *M. rosae*. DNA was extracted from *in vitro*-grown mycelia of 11 single spore isolates grown on PDA medium. High levels of polymorphism were detected among the isolates. They clustered into three distinct groups: Group 1consisted of isolates from eastern North America plus a European isolate (Germany), Group 2 included isolates from southern Sweden, and Group 3 included the isolates from Manitoba, Canada. The greater similarity of the environmental conditions in eastern North America and Europe as compared to the Canadian prairies suggest that climate and weather could be key factors in influencing the potential race structure of *M. rosae*. However, variations among closely situated sites, e.g. southern Sweden, also occurred.

Key words : blackspot, *Diplocarpon rosae*, garden roses, single spore isolate

Introduction

Blackspot is often regarded as one of the most severe diseases in garden roses (*Rosa hybrida* L.). This disease is caused by the fungus *Marssonina rosae* (Lib.) Died. (imperfect stage of *Diplocarpon rosae* Wolf, ascomycetous affinity) and is very widespread where the hosts are cultivated or growing wild. The genus *Marssonina* belongs to the class Imperfect fungi (Deuteromycetes), order Melanconiales and has an asexual life cycle with production of conidia in subcuticular acervuli. However, a sexual life cycle is occasionally observed with production of ascospores in apothecia (*Bolton & Svejda*, 1979).

Breeding for resistance to blackspot in roses has become one of the major goals in several rose breeding programs around the world since legal restrictions and consumer concerns about the application of fungicides have increased in recent times. However, resistance to blackspot has proved difficult to achieve, probably because of the many existing variants of *M. rosae*. A deeper knowledge about the genetic variation of the races and their geographic distribution is necessary for the development of a successful resistance breeding program.

Large variation in *M. rosae* isolates from differert rose cultivars and geographic locations has been reported by several authors. *Wenefrida & Spencer* (1993) found differences in spore size, colony color and pathogenicity, and *Jenkins* (1955) claimed that he identified both physiological races (differences in optimum temperature and pH) and pathogenic races (differences in average spot size and development of spots regardless of size respectively) in pure cultures of the pathogen. However, he most certainly used polysporous isolates and not single spore isolates for his inoculation studies. *Bolton & Svejda* (1979) found that lesion size was extremely variable within each isolate and host, and found evidence suggesting the presence of three races in their study. In a more recent study, *Debener* et al. (1998), could identify five different races among 15 single conidial isolates of the pathogen when ten plant genotypes were inoculated.

To our knowledge, variation between single spore isolates from the blackspot fungus has, with one exception so far, only been investigated with the classical biological pathotyping technique. With this technique, a set of different host genotypes is used for infection with different single spore isolates. A group of pathogens (isolates) that infect a given set of plant varieties is defined as a race (*Agrios*, 1978). The first investigation of genetic variation in *M. rosae* performed with molecular methods was reported by *Lee* et al. (2000). By using restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region on rDNA they were able to differentiate ten isolates of the fungus into three groups. However, the information of this work is very limited.

Various molecular methods have been used for studies of genetic variation in other fungi. Weising el al. (1991) detected high levels of polymorphism in *in vitro*-grown

mycelia of purified isolates of *Ascochyta rabiei* using DNA fingerprinting with synthetic oligodeoxynucleotides. *Kerssies* et al. (1997) found large genetic differences among isolates of *Botrytis cinerea* isolates sampled inside and outside a glasshouse when studied with random amplified polymorphic DNA (RAPD) analysis. Genetic variation in *Fusarium* species was investigated using RFLP analysis of single-spore lines (*Nicholson* et al., 1993). When studying genetic variability of *Colletotrichum lindemuthianum* in wild populations of common bean, *Sicard* et al. (1997) found higher levels of polymorphism with the RAPD approach compared to using RFLP of PCR-amplified ITS regions. The RAPD technique has in several studies proved to be fast and easy to use, and, if reproducibility can be achieved, sufficiently reliable. It furthermore has the advantage of not requiring any prior knowledge of the DNA sequence needed, which makes it very useful for studies of species that are not well known.

The present paper represents an initial RAPD based survey of genetic diversity among and within geograhically diverse groups of single-spore isolates of *M. rosae*.

Material and methods

Fungal material

Eleven single spore isolates of *M. rosae*, originating from four different countries, were included in this study (*Table 1*). Four of the 11 single spore isolates were represented by two subsamples each (referred to as A and B, or I and II, depending of origin, in *Table 1*) for a total of 15 samples. All isolates are now in the culture collection of the third author (CGD).

Preparation of single-spore isolates

Upon receipt of the infected leaves from abroad or collected from the rose field plots at the Agriculture and Agri-Food Canada (AAFC), Morden Research Station, Morden, Manitoba, Canada, the leaves were surface sterilized and incubated in petri plates with sterilized moistened filter paper to maintain high humidity. Spores washed from the leaves were collected approximately 7-10 days later and subsequently transferred to sterile petri plates with PDA (potato dextrose agar) and streptomycin. In a step-wise process, single spores were isolated from the plates and transferred to fresh PDA. Cultures grew for a period of approximately three months and subsequently transferred to glass beads for long term storage above liquid nitrogen. Prior to DNA extraction, the cultures were brought back to room temperature and re-plated on PDA and grown for approximately 3 months in an incubator (20-22°C). Spore suspensions of 11 single spore isolates were spread on PDA in November 2000. The isolates were sent to Balsgård, South Sweden, from AAFC, Morden, in January 2001, where they were maintained in a growth chamber in 23 ±1°C, 16 hours photoperiod and 50 µmol m-2 s-1. After approximately 2 weeks, small cubes of agar with mycelia were cut from the outer edges of the colonies and transferred to new PDA plates. Each single spore isolate was subcultured into 1–2 separate plates, referred to as A and B in *Table 1*. The subcultures were kept under the same growth chamber conditions as described previously.

Table 1. Single spore isolates of Marssonina rosae listed according to geographic origin

Original code	Origin	Plant hardiness zone (USDA)
99003 A	Morden, Manitoba, Canada	3
99003 B	Morden, Manitoba, Canada	3
99006-2	Morden, Manitoba, Canada	3
99037-2	Willowdale, Ontario, Canada	5
99038-3	Ottawa, Ontario, Canada	5
99058 I	Seeleys Bay, Ontario, Canada	5
99058 II	Seeleys Bay, Ontario, Canada	5
99008	Montreal, Quebec, Canada	5/6
99073-1	Washington DC, USA	7
99087-1	Pinneberg, Germany	7/8
99032-1 A	Balsgård, Scania, Sweden	7
99032-1 B	Balsgård, Scania, Sweden	7
99035-3	Stoby, Scania, Sweden	7
99034-1 A	Stoby, Scania, Sweden	7
99034-1 B	Stoby, Scania, Sweden	7

DNA extractions

Three and a half month after subculturing, mycelia was harvested from the PDA plates. As much agar as possible was removed from the mycelia and each isolate was adjusted to a maximum weight of 100 mg. In case of large fungal colonies, the material from each plate was divided into two subsamples, referred to as I and II in *Table 1*. The material was immediately frozen to -80°C after harvesting using a low temperature freezer. One week later, the samples were ground in liquid nitrogen to a fine powder using a mortar and pestle, and DNA was extracted with QIAGEN DNeasy Plant Mini Kit (Qiagen Inc.) according to the manufactors instructions.

DNA amplifications

The DNA amplifications were carried out in a 25 µl reaction mixture containing 2.0 mM MgCl2 (Advanced Biotechnologies), 0.3 µM primer (Operon Technologies), 1X buffer solution No. 4 (Advanced Biotechnologies), 0.2 mM nucleotide mix (Roche), approximately 10 ng genomic DNA and 1 U Taq polymerase (Advanced Biotechnologies). The mixture was subjected to an initial denaturation period at 94 °C for 5 min in a MJ Research thermocycler, and thereafter amplification for 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, followed by a 7 min extension time at 72 °C. The fragments were separated by electrophoresis in 1.8% agarose gels containing ethidium bromide and using a TPE (Tris-phosphate-EDTA) buffer system. The band patterns

were visualized by UV-light and photographed for further analyses. Molecular Weight Marker VI (Roche) was used to determine the size of the DNA fragments. The bands were scored manually as present or absent and recorded as 1 or 0, respectively. Some samples were amplified at least twice to ascertain reproducibility.

Pairwise comparisons between the individual samples were conducted using Jaccard's similarity coeffiecient, and a cluster analysis based on average linkage between groups (UPGMA) was performed. A dendrogram shows how the individual samples clustered. The resolving power of the primers was measured according to *Prevost & Wilkinson* (1999) as Rp= ΣIb where Ib (band informativeness) is defined as 1–[2x (0.5–p)], with p being the proportion of the 15 samples containing the polymorphic band.

Results

A total of 56 10-mer primers were screened for polymorphism in four DNA samples of the fungi, one single spore isolate each from Germany, Montreal, Morden, and Southern Sweden. Nine primers which generated polymorphic and reproducable amplification patterns were selected for analysis (*Table* 2). The selected primers produced from one to eight polymorphic bands which ranged in size between 450 bp and 2100 bp. A total of 35 polymorphic bands was produced which gives an average of 3.9 bands per primer. The resolving power of the primers varied between 1.32 and 9.02 (Table 2) and was highly correlated to the number of polymorphic bands. It is noticable that the CG content of the primers producing polymorphic bands was 67% whereas the AT content was only 33%.

Table 2. Selected primers, number of analysed polymorphic bands and resolving power

Primer ID	Sequence	No. of polymorphic bands	Resolving power
OPA-02	TGCCGAGCTG	5	5.72
OPA-04	AATCGGGCTG	2	2.66
OPA-09	GGGTAACGCC	7	7.86
OPA-14	TCTGTGCTGG	3	4.94
OPB-06	TGCTCTGCCC	2	2.64
OPE-02	GGTGCGGGAA	2	2.24
OPI-06	AAGGCGGCAG	1	1.32
OPI-07	CAGCGACAAG	8	9.02
OPI-14	TGACGGCGGT	5	5.46

The performed cluster analysis showed the 15 samples clustered into three distinct groups (*Figure 1*). Group 1 comprised all samples from Eastern North America together with the single sample from Pinneberg, Germany, group 2 comprised the samples from Southern Sweden, and group 3 comprised the three isolates from Morden, Canada. The three groups were well separated where group 3 turned out to be the most isolated. Of the three Morden samples, two from the same PDA plate but from different colonies showed a band difference of a major band with one primer (OPA14). This primer was tested in four different PCR runs with the same result. The third Morden sample, did not show any band differences with the first sample.

Some morphological differences were noted between the different colonies, but not further investigated.

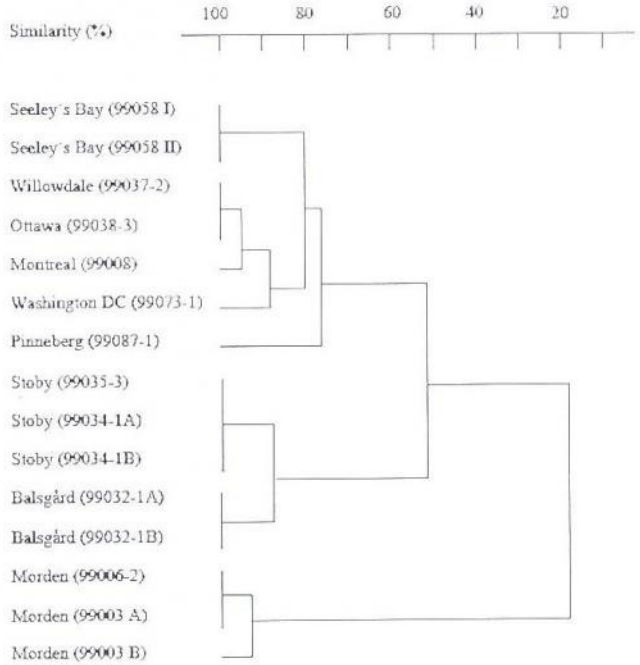

Fig. 1. Dendrogram based on UPGMA analysis of genetic similarity estimates among single spore isolates.

Discussion

Several studies of blackspot on roses have been concerned with the ability of the fungus to form lesions on different rose genotypes. A number of factors influence the results of such evaluations, and discrepancies sometimes occur between different published reports about the sensitivity to the disease in some genotypes. The ocurrence of several races of the blackspot fungus might be one explanation to such discrepancies. *Bolton & Svejda* (1979) showed a breakdown in blackspot resistance in the highly resistant *R. rugosa* hybrid cv. 'Martin Frobisher' in an inoculum trial, which they interpreted as a change in pathogenicity in the fungus. The same inoculum was unable to infect two otherwise generally very susceptible rose cultivars, thereby suggesting a new race of blackspot. Debener et al. (1998) also noted a susceptibility of a *R. rugosa* genotype towards blackspot, and made the same conclusion as *Bolton & Svejda* (1979) i. e. a development of the fungus, making it possible to infect the previously thought resistant genotype. *Castledine* et al. (1981) discussed the possibility of differences in the rose leaves, thereby making them different susceptible to fungus attacks. They also found the wild growing *R. arvensis* to be highly susceptible, although it had previously been thought to be

free from blackspot. A possible explanation could be that this *Rosa* species generally have a solitary growth, and that it is usually never in contact with the fungus.

The isolates from first group in our study, were all collected from the Eastern part of North America except one isolate from Germany, a rose garden in Pinneberg. The isolate from Washington DC was collected on a Hybrid Tea rose (cultivar unknown) near the White House and the one from Ottawa was obtained from AAFC, Central Experimental Farm, which has been a site for rose breeding for over 100 years. The isolate from Montreal originates from the Montreal Botanical Garden to which many rose cultivars and species have been imported during the years. Many plants have been imported from Europe (Germany) to Canada and vice versa. Normally, *M. rosae* spreads mainly by conidia dispersed in rainwater. The conidia are disseminated by splashing water, by people during cultivation, or by contact with sticky body parts of insects. Fallen leaves blown by the wind may disperse the pathogen locally, but conidia are usually airborne only in water drops (Horst 1983), which means that blackspot infection is usually more severe in rainy summers. However, the international movement of nursery crops in general and roses specifically are effectively spreading different fungal isolates and races. One could speculate that the placement of the German isolate among the North American ones, was that its host plant had been imported to Germany from Canada rather recently, and therefore still had the original infection. Transportation of spores have been observed previously within e.g. the fungus *Sclerotinia homoeocarpa*, dollar spot, which is a major disease on turfgrass. The fungus occurs frequently in Eastern USA, but was not reported until 1991 in the state of Oregon. In a RAPD study made on 26 different isolates of *S. homoeocarpa*, one isolate from Oregon was indistinguishable from another isolate collected in Connecticut showing the ability of the fungus to transport, probably on stray plants, over great distances (*Raina* et al. 1997).

The isolates in group 2, consisted of three samples with two of them divided into two subsamples, from two locations in the south of Sweden, with a distance of 30 km between them. There was only three bands which separated the samples from the two locations from each other. The infected leaves from which the single spore isolate from Balsgård was collected, belonged to a seedling ('Queen Elisabeth' X *R. pimpinellifolia* 'Flora alba plena') grown together with several other roses, mainly hybrids from a breeding program. The two single spore isolates from Stoby originated from infected leaves collected from two different unknown cultivars in a private garden. Considering the limited capacity for *M. rosae* to disperse, the existence of differentiation in RAPD profiles between samples collected in geographically close areas could indicate different races. However, differences between isolates of other fungi have been found at even much closer ranges. *Nicholson* et al. (1993) showed that more than one clone of a species of *Fusarium*, which causes ear blight on cereals, were present on a single spikelet of a plant. Previous inoculation tests have also shown that different genotypes within a rose species can be more or less susceptible to *M. rosae* (*Palmer* et al. 1966). In several studies, the species *R. wichuraiana* is considered to be comparatively resistant towards blackspot (*Castledine* et al 1981, Reddy et al. 1992). However, *Debener* et al. (1998) showed differences in susceptibility between two different genotypes of this species so that one of them was susceptible to all of the five isolates tested, whereas the other had no infections at all.

The third group, Morden, is very well separated from the other two groups. In Morden, rose breeding has been conducted for over 50 years. Over that time period, many new roses have been imported for testing and used as germplasm in the breeding program, just as in the Ottawa rose breeding station. Morden is located in the prairie area in Southern Manitoba. The climate is typical for the prairies, with dry, hot summers and very cold winters, whereas the other collection sites in the study (Europe and Eastern North America) have milder winters and cooler summers. Selection for fungus that can survive and grow in different types of climate can be presumed. The climate in Morden may result in a different selection pressure since the distribution of the pathogen is restricted by lack of rain, low humidity and high summer temperatures (*Baker & Dimock*, 1969). The low winter temperatures in Morden may lead to a selection of isolates more resistant to winter injury. The climate may also influence the occurrence of different races in an indirect way. At Morden, mainly very hardy roses are grown whereas a wider range of cultivars/species (more tender) can be grown at the other sites in our study. Several studies show that different isolates are able to infect different cultivars and species of roses (*Drewes-Alvarez*, 1992; *Debener* et al. 1998), and it has also been shown that differences in isolate pathogenicity depend on the cultivar (*Wenefrida & Spencer*, 1993). Therefore, as the type of host plants available in Morden are different from the plants at the other sites, a different set of fungi might be expected. Of the three isolates from Morden, that were analysed in our study, two were subsamples from the same isolate (99003 A and 99003 B). One of them lacked a band, present in the other subsample and in the third Morden sample when amplified with primer OPA-14. The amplification was performed four times with the same result. The band difference may be the result of a point mutation, although those usually are more or less impossible to identify with RAPDs.

The morphology of the different single spore isolates grown on PDA varied between some of our isolates. Since we do not know which cultivars or species most of our single spore isolates originate from, it is impossible to ascertain any connections between morphology of the colony and the host plant in our study. There are however, reports that colonies of *M. rosae* could differ both in color and morphology (*Lee* et al., 2000; *Wenefrida & Spencer*, 1993).

Infection of differential host genotypes by the single spore isolates used in our study, would be needed to ascertain a possible differentiation of pathogenic races in our material. *Weising* et al. (1991) showed that DNA fingerprint data

supported their classification of *Ascochyta rabiei* (pathogen of chickpea) isolates according to their aggressiveness in a biological pathotyping using a differential host genotype set. However, *Kerssies* et al. (1997) could not detect any relation between RAPD pattern and pathogenicity when genetic variation in *Botrytis cinerea* isolates were studied with RAPD analysis. When races of *Colletotrichum lindemuthianum* were characterized by RAPD by *Vilarinhos* et al. (1995), the races separated into groups that were different from those defined by the use of common bean differentials. The discrepancies were explained by the fact that while the definition of a pathological race by a set of differentials involve very few loci in the fungal genome, the RAPD technique allows the simultaneous comparison of a large number of loci.

Conclusion

The results in our present study indicate that the RAPD technique is useful in distinguishing different genotypes of *Marssonina rosae*. Even though the sample size is quite small and the number of primers used are limited, the results do show that there is considerable genetic differentiation between geographically distant collection sites but also some differences between relatively close areas (Southern Sweden). In a study of the genetic variability of the imperfect fungus *Colletotrichum lindemuthianum* in five wild populations of common bean, *Sicard* et al. (1997) found that 95% of the total genetic variation resided at the intrapopulation level. Therefore it would be of interest to examine the genetic variation of *M. rosae* more closely within a smaller area, like a rose field with different cultivars and species, or even on isolates collected from the same rose plant.

Acknowledgements

The authors are indebted to Robert Conner, Allen Xue, Dawn Chi, Barbara Dyck and numerous summer students for assistance. Thanks are expressed to the various cooperators in Canada and Europe who provided the samples. We are grateful to Hilde Nybom for her constructive criticism on the manuscript. This research was supported by grants from The Swedish Research Council for Forestry and Agriculture (SJFR).

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