RESISTANCE BREEDING FOR POWDERY MILDEW (Podosphaera pannosa) AND BLACK SPOT (Diplocarpon rosae) IN ROSES

ir. LEEN LEUS
"Les hommes de chez toi, dit le petit prince, cultivent cinq mille roses dans un même jardin...
et ils n'y trouvent pas ce qu'ils cherchent...
Et cependant ce qu'ils cherchent pourrait être trouvé dans une seule rose ou un peu d'eau..."

Le Petit Prince
Antoine de Saint-Exupéry
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RESISTANCE BREEDING FOR POWDERY MILDEW (*Podosphaera pannosa*) AND BLACK SPOT (*Diplocarpon rosae*) IN ROSES

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For the degree of Doctor (PhD) in Applied Biological Sciences:
Agronomy
Dutch translation of the title:
VEREDELING NAAR RESISTENTIE TEGEN ECHTE MEELDAUW (*Podosphaera pannosa*) EN STERROETDAUW (*Diplocarpon rosae*) BIJ ROZEN

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**Keuze ouderplanten**

*Veredeling is iets voor halfgoden*

Het is ondertussen meer dan 25 jaar geleden, maar ik weet nog hoe we thuis in de serre de anjers letterlijk oprolden om plaats te maken voor snijrozen. Sindsdien gingen gesprekken aan de keukentafel vaak over ziekten en plagen zoals echte meeldauw; witziekte, zoals dat bij ons heette. Ook schadedrempels werden besproken, al werd ook dat pas tijdens mijn studies zo genoemd en was dat bij ons thuis gewoon ‘spuiten of niet spuiten’. Ik ga hier niet verklaren dat het voor dit doctoraatsonderwerp daar allemaal begonnen is, dat ik daar ‘iets’ zou mee doen, zo ging dat in werkelijkheid niet. Al ligt de basis voor mijn interesse in tuinbouw allicht thuis. Veredeling was toen zeer ver van mijn kinderbed. Ik had wel gehoord dat er mensen waren die nieuwe rozen maakten. Nieuwe rozen creëren!? In mijn fantasie kon dat enkel het werk zijn van halfgoden, ergens ver weg.

**Bestuiving**

*Een schot in de roos*

Eerlijk gezegd, ik was een slecht voorbereide sollicitant op het (toen nog even) RvP. Welke vacature er was, wist ik eigenlijk niet goed. Iets met onderzoek, dat interesseerde me zeker wel. En over veredeling had ik ooit wel gesproken, maar dit was vast iets ‘cel en genachtig’, daar had ik toen nog niet veel kaas van gegeten. Niet echt voor mij dus, vreesde ik. Pas tijdens het sollicitatiegesprek werd me duidelijk dat het over rozenveredeling ging. De halfgoden waren plots onderzoekers van vlees en bloed, vlakbij in Melle! Dit was de start van dit doctoraatsonderzoek. Erik Van Bockstaele wil ik bedanken om dit onderzoekswerk op het DvP te mogen uitvoeren. Je inspanningen om projecten zoals dit op te starten en te steunen, zorgen voor de aanwezigheid van een uitgebreide staf en onderzoeksinfrastructuur. De aanwezigheid van zoveel ervaring en faciliteiten ten behoeve van jonge onderzoekers is een ongelooflijke luxe. Zo kon ik aan de slag met verschillende technieken: van klassieke kruisingsveredeling, over moleculaire merkers en flow cytometrie tot transformaties, … Daarnaast bewaar ik ook de beste herinneringen aan vele andere activiteiten die ik mocht uitvoeren, zoals het deelnemen aan Agribex in 1998, het maken van een lespakket over biotechnologie, het werken met de flow cytometer op azalea, gras … Op die manier kreeg ik ook de kans veel te leren over de veredeling van andere gewassen. Bedankt daarvoor!

Toen ik op het DvP begon en zag hoeveel technieken er werden uitgevoerd, vreesde ik dat nooit allemaal te kunnen leren. En inderdaad, ik leerde dat ik dat niet allemaal kan kennen. Maar hulp was er genoeg om me dingen te leren en hulp te bieden waar nodig. Aanvankelijk maakte ik via Marleen en Jacky kennis met de rozenveredeling en bij Marc kon ik terecht voor wat het labo betrof. Ik kon ook steeds een beroep doen op de laboranten om me te helpen. Ik vrees hier mensen te vergeten, want ik denk dat er bijna geen laborante op het DvP te vinden is die door mij nooit met vragen werd bestookt of om hulp werd gevraagd. Van Annique, Sofie en Kristien leerde ik in het begin veel over het in-vitrowerk, later kwam daar ook Ronald bij. Voor flow cytometrie kon ik een beroep doen op Hilde. Met vragen over DNA-werk kon ik terecht bij Arianne, Katrien, Laurence, Nancy, Veerle(s), … en alle anderen. Ook Annemie wil ik bedanken voor het wassen van zoveel vuile potten en
proefbuizen. J’ai aussi eu beaucoup d’aide de Fabienne pendant la période où elle a travaillé au DvP. Merci! Ook op collega’s onderzoekers kon ik steeds een beroep doen met vragen over computer- en andere problemen. Velen onder hen gingen mij voor in de afronding van hun doctoraat. Ook hier is het gevaarlijk namen te noemen en te vergeten, toch een woord van dank voor Angelo, Ellen, Els, Evelien, Hilde, Inge, Isabel(le(s)), Jan, Katrijn, Tom, Veerle, … en Johan die steeds mijn mentor was bij de praktische uitvoering.

**Zaadzetting**

**Veredeling staat zelden op zichzelf**

Dit onderzoek kreeg pas zijn definitieve ziekteresistentie-richting vanaf 1999 dankzij Monica Höfte. De inbreng van Monica en de mensen van het Labo voor Fytopathologie is steeds belangrijk geweest doorheen dit werk. Zowel bij het schrijven van projecten, het opvolgen van resultaten en de wetenschappelijke invulling en afwerking van dit doctoraat heeft Monica een grote rol gespeeld. Monica, je combineerde hierbij wetenschappelijk inzicht met psychologisch doorzicht om mijn “intuitieve, associatieve” verstand in de juiste richting te sturen. Voor mij was dit een positieve stimulans om dit werk tot een goed einde te brengen. Ondanks dat het praktische werk meestal werd uitgevoerd op het DvP, heb ik me altijd zeer welkom gevoeld in het Labo voor Fytopathologie. Hartelijk dank allemaal voor jullie hulp bij het uitdenken van inoculatietesten, induceren van resistentie, scoren van ziekteresistentie en verwerken van resultaten van fytopathologisch onderzoek. De seminaries die jullie onder de middag organiseren hebben mij veel geleerd over plantenziekten en onderzoeks-methodologie.

**Zaailingen**

**Veredeling is meer dan kruisen**

Tijdens deze studie hebben we nooit de luxe gekend (misschien maar goed ook) te werken met schimmels die je gewoon op plaats kan groeien. Ik kan het me zelfs nauwelijks voorstellen dat het zo eenvoudig kan gaan. Ontzettend veel tijd diende dan ook te worden geïnvesteerd in het onderhouden van schimmelisolaten op in-vitroplanten, de in-vitrorozen zelf, … Zonder de hulp van Nathalie hadden deze onoverkomelijke taken weinig tijd gelaten voor het eigenlijke onderzoek. Omdat we elk andere voorkeuren hadden bij de uit te voeren taken, waren we zeer compatibel bij de uitvoering van het project. Het bouwen van curieuse hoge piramides van petri-platen is voor mij nog steeds een onbegrenpt en onuitvoerbare labo-discipline. Nathalie, bedankt voor de hulp bij het eindeloos overzetten van in-vitroplantjes, schimmelculturen, scoren van planten, kweken van bacterieculturen, uitvoeren van DNA-werk, …

**Selectie**

**Het is eenvoudiger een lelijke gezonde roos weg te gooien dan een mooie zieke**

Veredeling is naar planten kijken. Scoren is een belangrijke bezigheid bij de selectie naar ziekteresistentie. In theorie gaat dat als volgt: het is mooi weer, Johan en ik lopen door een veld vol rozen en noteren waar er ziektes optreden en waar niet. De praktijk is echter anders. Je kunt er bijna zeker van zijn, wanneer we scoren op het PCS met Filip Rys en Frans Goossens, dan regent het. Op het DvP is het bij het scoren meestal bloedheet. Rozen spreken tot de verbeelding. Iedereen weet hoe
vaak ze bezongen en bedicht zijn, wees dus beducht! Misschien komt het ook door
de hitte, maar het is moeilijk geconcentreerd te blijven tijdens het scoren. De rozen
proberen ons van hun zieke bladeren af te leiden met mooie of bijzonder rare en/of
lelijke bloemen, wat leidt tot allerhande gefilosofeer. We verzinnen dan bijvoorbeeld
namen voor onze kandiaat cultivars zoals ‘Tof in den Hof’ of ‘La Tristesse du Jardin’,
al naargelang de schoonheid van de plant. Ondertussen wordt Johan afgeleid door
zijn Buddleja’s in de verte, ‘zijn ze allemaal even paars’?, terwijl ik zelf de
biodiversiteit aan lieveheersbeestjes op onze planten bewonder. Ik vraag me af of
dat bij pakweg de selectie van gras ook zo gaat.

Vegetatieve vermeerdering

Veredeling is boekhouden
Het lijkt eenvoudig, wat plantjes kruisen, zaaien, in de serre zetten en later op het
veld. Jaja, totdat je wil weten wat de ouders zijn van deze of gene kruising, of tot je
je afvraagt waar de zaailingen die 3 jaar in de serre zo mooi was naartoe is. Want,
veredeling is boekhouden! Je maakt duizenden kruisingen, houdt bij voorkeur bij wat
je wanneer hebt gekruist, wie de ouders zijn, vervolgens oogst je de bottels, tel je de
zaden, noteer je welke zaden van welke ouders zijn, zaai je de zaden uit, nog steeds
wetend wie wie is, je verplant een 10 000-tal zaailingen op naam in de serre, de
enkele honderden die er na een jaar serre nog overblijven vermeerder je en plant je
weer onder de juiste naam op het veld, en dan hoop je maar dat er geen gele bloem
opduikt in een veldje rode. Ondertussen staan er weer duizenden zaailingen klaar in
de serre en voer je opnieuw honderden kruisingen uit. Enkele jaren geleden hoorden
we op het Eucarpia symposium een gedurfde, maar eerlijke uitspraak over de
naamgeving in plantentuinen “30% of the plants is mislabbeled”. Sindsdien is dit een
gevleugelde uitspraak wanneer er iets niet klopt. Maar het lukt aardig deze stelling te
verwerpen, dankzij de mannen van buiten: Jo, Frederik en Roger. Ik heb veel
bewondering voor het geduld en de ernst waarmee jullie die stroom van duizenden
planten managen. Ik wil jullie daarnaast ook van harte bedanken voor jullie
vakkundige verzorging van de planten, het uitvoeren van de kruisingen, bouwen van
tenten ...

Rozenconcours

Rozen zijn geen modelplanten
Rozen zijn geen modelplanten, nee, rozen staan daar ver boven. De roos is niet voor
niets de meest ver’edel’die sierplant; zij beweegt zich in adelijke kringen. Dankzij
Frans mocht ik ook kennismaken met dit aspect van de roos, het mondaine
rozenleven op rozenconcours, prinselijk bezoek, ...
Andere plezierige momenten tijdens het werk beleefde ik met mijn DvP-
bureaugenoten, aanvankelijk Ilse en Sandra, en later Tom en Katrijn. Of het nu over
planten of wielrennen ging, ik kon altijd iets van mijn collega’s opsteken. Ook
vrienden en familie wil ik bedanken voor hun aanmoedigingen en interesse in mijn
rozenwerk en de aangename momenten de voorbije jaren.

Commercialisatie

Op rozen zitten
Er is geen onderzoek mogelijk zonder dat er middelen voor zijn. Dit onderzoek werd
aanvankelijk uitgevoerd via een project van het Ministerie van Middenstand en
Landbouw (DG6), later overgenomen door IWT-Vlaanderen. Hartelijk dank voor de jarenlange financiële steun en het vertrouwen in de projecten die uiteindelijk tot dit werk hebben geleid.

**De rozentuin**

*Rozengeur en maneschijn*

Wanneer ik nadenk over het voorbije jaar, zou ik over een soort van sabbatjaar kunnen spreken. Niet in de betekenis van een jaar niets doen, maar een jaar waarin ik andere dingen deed dan de activiteiten in de voorgaande jaren. Het onderzoek in de serres en in het labo maakte grotendeels plaats voor het schrijven van dit werk. Dit ‘andere’ jaar kwam er nadat ik in oktober 2004 door Paul Van Assche aan het departement Biot van Hogeschool Gent werd aangeworven als academiserings-assistent. Ik vond er de ideale omstandigheden om het werk van de afgelopen jaren te verwerken en uit te schrijven tot dit doctoraat. Ik bedank mijn collega’s Filip, Geert, Joris, Omer, Stefaan en Steven om mij de ruimte te geven alles gecomputeerd te krijgen en tegelijk op tijd voor afleiding en entertainment te zorgen. Ik ben blij nu dit schrijven er op zit, terug ‘echt’ aan de slag te kunnen gaan.

Als kind wou ik graag schrijfster worden, dat leek me toen een eenvoudige bezigheid. Bij de afronding van dit werk weet ik dat ook hier de waarheid anders is. Voor de afronding van dit werk wil ik nogmaals Johan bedanken, voor het bemannen van mijn crisissecretariaat en het maken van grafieken met zijn speciaal programma’tje.

Tot slot ook een woord van dank voor de leden van de examencommissie: Prof. dr. Thomas Debener, Dr. Tik de Vries, Prof. dr. ir. Georges Hofman (voorzitter), Prof. dr. ir. Dirk Reheul, Prof. dr. ir. Els Van Damme (secretaris), Dr. ir Johan Van Huylbroeck en Prof. dr. ir. Marie-Christine Van Labeke voor het nalezen van dit werk en suggesties voor de uiteindelijke afwerking.

Ook al worden er thuis geen rozen meer geteeld, toch hoop ik dat enkele van de kandidaat cultivars uit dit onderzoek de weg vinden naar de telers, om te worden gebruikt in openbaar groen en in talrijke tuinen. Met dank voor al jullie hulp!
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Samenvatting

In het verleden lag de nadruk bij rozenveredeling op externe plantkwaliteit zoals bloemkleur, -grootte en -vorm, stengelopbrenst, doornloosheid en planthabititus. Weinig aandacht was er voor ziekteresistentie. De meest belangrijke schimmelziekten op tuinrozen zijn sterroetdauw (Diplocarpon rosae) en echte meeldauw (Podosphaera pannosa). Dit doctoraatsonderzoek had tot doel het ziekteresistentieveredelingsprogramma voor tuinrozen te ondersteunen via het aanreiken van kennis en het ontwikkelen van praktisch bruikbare kruisings- en selectiemethoden. Belangrijk hierbij was het verwerven van kennis over de pathogenen zelf, het evalueren van bio-toetsen, en het testen van de mogelijkheden van interploïdie kruisingen en geïnduceerde resistentie.

Kennis over de pathogenen is belangrijk bij het screenen naar ziekteresistentie. Voor beide ziektes werden pathotypes met verschillende virulentie tegenover verschillende rozen- en Prunus-genotypes gevonden. Sommige echte meeldauw isolaten zijn ook virulent voor Prunus avium. Deze echte meeldauw types zijn te herkennen aan één basepaar verschil in de ITS sequentie vergeleken met de sequentie van isolaten die enkel op roos of enkel op Prunus kunnen groeien.

Om genotypes met een betere ziekteresistentie te selecteren zijn geschikte bio-toetsen nodig. Inoculatietests op afgetrokken blad zijn bruikbaar voor het herkennen van geschikte ouderplanten met een goede ziekteresistentie. Water moet worden vermeden wanneer wordt gewerkt met conidia van echte meeldauw. Daarom wordt voor echte meeldauw een inoculatietoren gebruikt. Voor sterroetdauw worden afgetrokken bladeren in een labotest geïnfecteerd met een conidiasuspensie.

Artificiële infectiemethodes op jonge zaailingen werden ontwikkeld voor beide ziekten. Inoculumplanten tussen de zaailingen verspreiden echte meeldauw vroeg en uniform in de serre, op deze manier worden resistenterere zaailingen snel herkend. Sterroetdauw resistentie werd getest door de zaailingen te bespuiten met een conidia-suspensie. Normaalgezien komt sterroetdauw niet voor in serres, maar het gebruik van een plastic tent en het begieten van de planten op het blad laat een artificiële infectie in de serre toe. Een combinatie van de screeningsmethodes voor beide ziektes in het eerste groeiseizoen van de zaailingen is mogelijk.

Er werden correlaties gevonden tussen echte meeldauw infecties op jonge zaailingen in de serre en veldinfectie tijdens de latere selectie. Het evalueren van sterroetdauw in het veld dient gespreid over verschillende jaren te gebeuren aangezien de schimmel de tijd nodig heeft om zich uniform over het veld te verspreiden.

Bij rozencultivars is er weinig resistentie tegenover schimmelziektes aanwezig. Om een verhoogde ziekteresistentie te verkrijgen worden rozenspecies ingekruist. Hiervoor zijn interspecifieke hybridisaties en daardoor vaak ook interploëdiekruisingen nodig. Veel rozensoorten zijn diploïd terwijl cultivars vaak tetraploïd zijn. Kruisingen tussen di- en tetraploïden leveren triploïde nakomelingen op. Veel triploïde rozen zijn
bruikbaar voor verdere kruisingen. Op deze manier is de introgressie van genetische kenmerken van diploïde species in tetraploïde cultivars mogelijk.

Door middel van rhizobacteriën, die de plantengroei bevorderen, en specifieke chemische stoffen kan resistentie in planten worden geïnduceerd. Dit onderzoek toonde aan dat BTH (een salicylzuur analoog), een extract van *Reynoutria sachalinensis* en verschillende specifiek rhizobacteriën resistentie tegenover echte meeldauw induceren bij rozen.

Resultaten uit dit doctoraatsonderzoek leidden tot een efficiëntere selectie naar ziekteresistentie bij rozen. In dit onderzoek geselecteerde rozengenotypes worden momenteel verder geëvalueerd als kandidaat cultivars.
Summary

In the past, priority in rose breeding was given to external plant quality like flower colour, size and shape, shoot yield, thorniness and plant habit. Little attention was paid to resistance to fungal diseases. In garden roses most important fungal diseases are black spot (*Diplocarpon rosae*) and powdery mildew (*Podosphaera pannosa*). The aim of this research was to support a disease breeding programme for garden roses by gathering knowledge and the development of crossings- and selection methodology useful in practice. It was important to study the pathogens themselves, to evaluate bioassays, and to test possibilities of interploidy crosses and induced resistance.

Knowledge on the pathogens is important in disease resistance screening. For both fungal diseases pathotypes differing in virulence towards different rose genotypes were found. Some powdery mildew isolates found on roses are virulent for *Prunus avium* too. These powdery mildew types can be recognised by a one base pair difference in the ITS sequence compared to powdery mildew isolates virulent only for rose or only for *Prunus* spp.

To be able to select genotypes with enhanced disease resistance in breeding and selection, appropriate bio-assays are needed. To select parent plants with a good potential for disease resistance, inoculations are made in a standardised way on detached leaves. For powdery mildew an inoculation tower can be utilised. Water should be avoided when conidia of powdery mildew are used. For black spot, an inoculum suspension can be used on detached leaves in a lab test.

Disease resistance should be regarded as one of the main characteristics in seedling selection and should be evaluated as soon as possible to be most efficient. Therefore artificial inoculations are made on young seedlings. For powdery mildew, inoculation plants in between the seedlings make infection spreads early and uniformly in the greenhouse. For black spot, greenhouse plants are inoculated by spraying a conidia suspension. Normally black spot does not occur in greenhouses. Artificial inoculation is possible when the plants are placed under a plastic tent to prolong the time of leaf wetness and when they are watered on top of the plants. A resistance screening for both diseases can be combined in the first growing season of the seedlings.

The occurrence of powdery mildew on rose genotypes in the greenhouse is correlated with the results of later field evaluation. Field evaluations on the occurrence of black spot should be made in different years, since the pathogen needs time to spread uniformly in the field.

Among rose cultivars, little resistance to fungal diseases is found. To enhance disease resistance, crosses with rose species are needed. Therefore interspecific hybridisations by interploidy crosses are necessary. A lot of rose species are diploid, whereas cultivars are often tetraploid. Crosses between di- and tetraploids yield triploid offspring. Many triploid roses can be used in crosses. In this way genetic material of diploid species can be introduced in tetraploid cultivars.
Resistance in plants can be induced by plant growth promoting rhizobacteria and specific chemical compounds. In roses, resistance to powdery mildew is induced with BTH (salicylic acid analogue), an extract of the giant knotweed (*Reynoutria sachalinensis*) and different plant growth promoting rhizobacteria. Possibly this induced systemic resistance can be used to recognise genotypes with partial resistance.

Results from this study have led to a more efficient selection for disease resistance roses. In this work selected rosegenotypes are currently evaluated for their potential as new cultivars.
Abbreviations and acronyms

%CV  coefficient of variation
2,4-D  2,4-dichlorophenoxyacetic acid
ALFP  amplified length polymorphisms
BAP  6-benzylaminopurine
BSA  bovine serum albumine
BTH  benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methylester (Bion®)
CFU  colony forming units
CLO  agricultural research centre
cv.  cultivar
DAPI  4′,6-diamidino-2-phenylindol
DF%  disease factor
DI%  disease index
DNA  deoxyribo nucleic acid
dNTP  deoxynucleotide triphosphate
DvP  department of plant genetics and breeding
EB  ethidium bromide
FDA  fluorescein-di-acetate
FDR  first division restitution
GA3  gibberellic acid
GISH  genomic in situ hybridisation
HR  hypersensitive response
IBA  indolebuteryc acid
INA  2,6-dichloroisonicotinic acid
ISR  induced systemic resistance
ITS  internal transcribed spacer
JA  jasmonic acid
MAS  marker assisted selection
MEA  malt extract agar
MEB  malt extract broth
MS  Murashige & Skoog (medium)
MS1B  modified Murashige & Skoog (medium)
n, 2n  respectively: gametic cell and somatic cell
NAA  α-naphtalene acetic acid
NBS  nucleotide binding site
NCBI  national centre for biotechnology information
OD  optical density
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PCS  proefcentrum voor sierteelt
PDA  potato dextrose agar
PGPR  plant growth promoting rhizobacteria
PI  propidium iodide
PR  pathogenesis related
PVP  polyvinyl pyrrolidone
QTL  quantitative trait loci
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RAPD</td>
<td>random amplified polymorphic DNA</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>RGA</td>
<td>resistance gene analogue</td>
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<tr>
<td>SA</td>
<td>salicylic acid</td>
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<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
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<tr>
<td>SDR</td>
<td>second division restitution</td>
</tr>
<tr>
<td>SH</td>
<td>Shenk &amp; Hildebrandt (medium)</td>
</tr>
<tr>
<td>sp.</td>
<td>species (singular)</td>
</tr>
<tr>
<td>spp.</td>
<td>species (plural)</td>
</tr>
<tr>
<td>SSR</td>
<td>simple sequence repeat</td>
</tr>
<tr>
<td>TDZ</td>
<td>thidiazuron</td>
</tr>
<tr>
<td>x, 2x, ...</td>
<td>number of chromosome sets</td>
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INTRODUCTION
OBJECTIVES AND OUTLINE OF THIS WORK

At the Department Plant Genetics and Breeding (CLO-DvP), both cut and garden roses have been bred since the sixties. Until now, more than 90 cultivars have been released. Since the end of the nineties, breeding and selection is focused on garden roses only. In recent years, more effort is put in breeding-supporting research and pre-breeding work resulting in a more research-based approach (Thomas et al., 2004). Breeding implies the use of yesterday’s gene pool, to create with today’s technologies cultivars of tomorrow. In ornamental plants, breeding for novelties often aims at new aesthetical properties. These are frequently gained through “luck”, driven by the breeders experience, intuition and creativity and in other cases by the application of “new” techniques. In garden roses - more than in cut roses were more objective criteria have to be taken into account - this intuition driven selection is still a very substantial part of the breeding work. Till recently, priority in breeding was almost always given to external plant quality exclusively which involves e.g. flower size, shape and colour, plant habit, thorniness and shoot yield (de Vries, 2000). The use of pesticides made disease resistance in plants less important. Nowadays, sustainable development, environmental concern, legislation and the consumer’s demand have piloted disease resistance to a top priority in plant breeding. Within this framework fungal disease resistance became the major objective in the DvP rose breeding programme. In roses, six main diseases occur: powdery mildew (Podosphaera pannosa), black spot (Diplocarpon rosae), leaf spots caused by Elsinoe rosarum, grey mould (Botrytis cinerea), rust (Phragmidium spp.) and downy mildew (Peronospora sparsa). All diseases mentioned can have a serious impact on rose plants, affecting growth of the plant, yield and aesthetical value or in worst case cause death of the plant. Powdery mildew and black spot are ubiquitous present on garden roses. The occurrence of other pathogens seems more dependent on environmental and climatological conditions. Therefore, this study is focussed on powdery mildew and black spot.

When it comes to objective characteristics like disease resistance, breeding should be based on knowledge of the pathogens, on detection of resistances in rose genotypes and on the application of standardised screening methods. Resistance breeding in roses is hampered by the lack of these tools. Therefore the goal of this work is the development of auxiliary methodology for fungal disease resistance breeding in roses.

Several aspects supporting the breeding work will be studied:
- A representative fungal disease screening requires knowledge on the pathotypes used. Inoculations with different pathotypes or a mixture of the most virulent ones can assist to recognise (partial) resistances in rose progenitors and progenies.
- In the classical rose breeding scheme, disease resistance screening on progenies only starts in the second year of evaluation. In the first year, seedlings are
evaluated in greenhouse conditions mainly for flower characteristics. Powdery mildew can occur in this first year of evaluation but black spot does not develop in greenhouses. Therefore black spot is only evaluated after the seedlings are propagated and transplanted in the field. In the field candidate cultivars are subjected to a variable, natural inoculation of uncharacterised pathotypes. A screening for disease resistance requires the development of standardised artificial inoculations for both progenitors and progenies. Because fungal disease resistance has become a major selection criterion, selection for this characteristic should be applied as early as possible in the selection process.

- Resistance in plants can be induced systemically by use of rhizobacteria, plant extracts and chemical compounds. More knowledge on the possibilities of induced resistance in roses is needed. It is possible that induced resistance can be used as a tool to recognise partial resistant plants useful for breeding (Hijwegen & Verhaar, 1995; Hijwegen et al., 1996).

- As known by breeders and found in literature, disease resistance in cultivated roses is generally low. Breeding for disease resistance based on a genetic approach is complicated in tetraploid and highly heterozygote rose cultivars. A broader germplasm has to be introduced from species through interspecific hybridisation. Interspecific hybridisation in roses often implies interploidy crosses. Most cultivars are tetraploid, whereas a lot of species are diploid. It should be examined whether triploids are a useful bridge in interploidy breeding in roses.

This dissertation is organised in 6 chapters. In chapter 1 the history of roses and rose breeding is described. Information on black spot and powdery mildew in roses is presented. In chapter 2, first of all the variation in the pathogens, powdery mildew and black spot is studied. This variation is tested by differential reactions on rose cultivars and species. For powdery mildew also sequence analysis is used to study genetic diversity within this fungal species. Bioassays for disease resistance screening are developed in chapter 3 for parent plants, seedlings and candidate varieties. These bioassays are tested for their practical use in rose breeding and selection. Chapter 4 describes induced systemic resistance in roses by application of resistance inducers like chemicals and plant growth promoting rhizobacteria. The possibilities for this type of resistance in practice are discussed for powdery mildew. Fungal resistance occurs in species, rather than in cultivars. However, ploidy differences between species and cultivars can hamper efficient crossbreeding. The possible use of triploids as a bridge for crosses of resistant diploids with tetraploid cultivars, is evaluated in chapter 5. In Chapter 6 the general conclusions of this work are given. Besides is evaluated how the developed methods, can be implemented in a programme for fungal disease resistance breeding in roses.
Chapter 1

LITERATURE REVIEW

In this chapter we run through literature on roses: the historical outline of rose species and cultivars is described and economical aspects are regarded. In view of the practical work in this study rose breeding and induced resistance are presented, an introduction on the rose diseases black spot and powdery mildew is given and breeding for resistance to fungal diseases is discussed. At the end of this chapter the objectives and the outline of this work on disease resistance breeding in roses are described.
1.1 General introduction

1.1.1 Classification of the genus *Rosa*

The Rosaceae is a large and diverse family of approximately 100 genera and about 3000 species. Of these species 90 are economical important such as apple, peach, strawberry, plums and roses. The Rosaceae are divided in four subfamilies according to the fruiting type: the Spiraeoideae, the Amygaloideae, the Maloidea and the Rosoideae with the genus *Rosa*.

**Taxonomy**

| Kingdom | Plantae - Plants |
| Subkingdom | Tracheobionta – Vascular plants |
| Superdivision | Spermatophyta – Seed plants |
| Division | Magnoliophyta – Flowering plants |
| Class | Magnoliopsida – Dicotyledons |
| Subclass | Rosidae |
| Order | Rosales |
| Family | Rosaceae – Rose family |
| Genus | *Rosa* L. – rose |

Originally roses are found in the Northern hemisphere only. The most accepted classification in Rosa was established by Rehder (1940) and contains approximately 120 species divided in four sub-genera of which *Hulthemia*, *Platyrrhodon* and *Hesperodos* contain each one species; the fourth subgenus *Eurosa* contains all true roses grouped in ten sections. Seven of these sections are of major importance in garden rose systematics: Pimpinellifolia (di- and tetrapioid), Chinensis (Indicae) (diploid), Gallicanae (tetrapioid), Cinnamonomeae with sometimes the Carolinae included (di- to octoploid), Caninae (tetra- to hexaploid) and Synstylae (diploid). The three other sections are very small, they consist of one or two species only, and have only limited importance in garden roses: Laevigatae, Banksiae and Bracteatae.

1.1.2 Origin of rose cultivars

Roses are the most ancient produced ornamentals and continue to be highly appreciated (Gudin, 1999). According to the interpretation by the author of the importance of species in rose domestication, only 8 to 15 species contributed to the original germplasm of the modern rose cultivars.

The widely accepted overview by Wylie (1954) mentions following important species:

- **Chinensis:** *R. chinensis*, *R. gigantea*
- **Gallicanae:** *R. gallica*, *R. damascena*
- **Synstylae:** *R. multiflora*, *R. wichuraiana*, *R. moschata*
- **Pimpinellifoliae:** *R. foetida*
Involved but of less importance:

- Cinnamomea: *R. rugosa, R. cinnamomea*
- Pimpinellifoliae: *R. pimpinellifolia*
- Synstylae: *R. phoenica, R. sempervirens, R. arvensis*
- Caninae: *R. rubiginosa*

Important characteristics were introduced in the rose cultivar gene pool from the progenitors of the modern rose cultivars, like recurrent flowering from *R. chinensis* (1800), cold resistance from *R. wichuraiana*, or yellow flower colour by *R. foetida* (1900). Of the important ancestors mentioned, only *R. gallica* and *R. foetida* are tetraploid, whereas the rest is diploid (Wylie, 1954). In contrast, most of the modern rose cultivars are tetraploid (de Vries & Dubois, 1996). Molecular work confirmed the narrow genetic background in modern rose cultivars (Matsumoto et al., 1998) and the influence of some ancestors in the germplasm of the rose cultivars (Leus et al., 2000 & 2004; Martin et al., 2001).

The number of rose cultivars on the market is unknown, although about twenty thousand are registered in Modern Roses XI (Roberts et al., 2003b) but many more cultivars than those listed are commercialised. Rose cultivars are commercially used as cut flowers, garden roses and miniature pot plants. Furthermore they are also one of the major flowers used for the perfume industry and they are important for their medicinal and culinary qualities (Gudin, 1999).

Traditionally, cultivars are classified as Hybrid Tea (one flower), Floribunda (cluster-flowered) and Miniature roses. Most cut rose cultivars are Hybrid Teas, whereas garden roses are often of the Floribunda type. But, the distinction between these original groups of horticultural classes including Polyanthas, Hybrid Teas, Floribundas and Miniatures has faded by intensive breeding. In fact breeding will tend to a narrowing of the original gene pool. Particularly in glasshouse cultivars it is often questioned if the genetic variation is sufficient to ascertain future breeding. It is suggested that rose selection for more than 100 years, based on a narrow genetic background, may have led to severe genetic erosion (de Vries & Dubois, 1996). Others oppose this thesis and refer to the high heterozygosity in the tetraploid cultivars and the vegetative propagation (Gudin, 2001; Noack, 2003). That does not alter the fact that in breeding a large gene pool is needed to maintain breeding work leading to valuable results.

Rose breeding and selection is already going on for centuries. Therefore it is not surprising that breeders emphasize now again the (re)use of the original gene pool. To some extent the original species involved in early rose cultivar development have been reused. For example *R. wichuraiana* and *R. rugosa* were used in the development of the amphidiploid *R. x kordesii* in 1952.
1.1.3 Economical importance and tendencies in roses linked to rose breeding

World-wide, yearly approximately 8 billion rose stems, 80 million potted plants and 220 million garden roses are sold (Roberts et al., 2003b). Most economic information on roses focuses on cut roses. Roses are still the absolute number one in cut flower production. Red coloured roses represent 30% of the market (Chaanin, 2003). Since the eighties, production areas are rising in tropical regions of Africa and America around the equator (Zieslin, 1996; Gudin, 1999). The consequences are new types of production according to climatic conditions and socio-economic factors (Zieslin, 1996). Cultivation in Latin America on high altitudes (2000-3000m) with cold nights asks for good low temperature growing ability. For breeding this has also led to new demands concerning transportation like post harvest quality and thornlessness. However, there is little information on rose plant physiology or development in these new areas. The main breeding companies have organised varietal testings in these areas (Gudin, 1999).

Cut roses are divided in classes according to the flower size. In the past, roses were a luxury product in winter; now, by new production areas and intensified production by traditional producers roses have become a product of mass consumption (Zieslin, 1996). This evolution is visible in rose types cultivated, whereas the rose as a luxury product was large-flowered, nowadays more cultivars with a high production of small flowers have appeared. For small flowered roses, the harvesting cost is 80% of the total cost, for these types there is no commercial benefit in countries with high labour costs (Chaanin, 2003). In the traditional production area of the Netherlands growers intensified their growing system and expanded their areas towards soilless cultivation, artificial lightening, high heating, CO₂ addition, climate regulation and robotisation. The use of new plant management techniques like plant bending and own rooted plants were studied. At the same time an enhanced renewal of varieties every three years is maintained whereas ten years ago this was five years. This has led to a very quick turn-over of the plants and varieties (Gudin, 1999).

Traditionally cut flower production in Flanders was situated around Aalst. In contrast to the Netherlands, in Belgium the augmented competition led to a tremendous decrease in the amount of growers and of the total production area. According to the analysis of Verwilt & Matthys (2002) the cut flower production centre around Aalst has nearly extinguished. Since 1989 the disinvestment in greenhouses in Belgian horticulture as a whole is tremendous. The main reason is a lack of attention in governmental policy for greenhouse horticulture, besides lack of strategy amongst the growers themselves. In cut flower production only some older growers with small and older greenhouse surfaces (on average 0.68 ha, with two thirds glasshouse area) still remain. Only a few growers will survive next decade in Flanders with larger and modern greenhouses. In the last years the area for cut flower production kept decreasing from 188 ha in 1980 (Van Lierde & Taragola, 1998) to 107 ha in 1998 and 89 ha in 2003, whereas the area for cut roses remained stable the last 5 years on about 44 ha. By this, half of the area for cut flower production consists of roses (Algemene directie statistiek en economische informatie, 2005).
New markets for cut roses on the still expanding market are exploited, among them new propagating countries like Mexico or South-Africa. Other countries forming new markets by import are the Persian Gulf states, Eastern Europe (Zieslin, 1996), ex-Soviet countries and Argentina (Gudin, 1999). India and China, tend to develop new production areas for their own consumption (Zieslin, 1996).

Less discussed in economic figures is garden rose production, which is often considered as nursery plants. In contrast to a growing interest of consumers in Belgium for gardening, which made nurseries flourish for the last 20 years, the production area of rose bushes in open air diminished from 295 ha in 1998 to 208 ha in 2003 (Algemene directie statistiek en economische informatie, 2005) although the production area for nursery plants increased with 10%. In 2004 the export value of Belgian roses was 4.1 million Euro; roses were mainly exported to France and The Netherlands (De Geest & Boerave, 2005). Main production areas in Belgium are situated around Wetteren and Lesdain. A large amount of the garden rose cultivars grown in Belgium are DvP-cultivars.

A growing concern for environmental preservation influenced garden rose breeding. Disease tolerance became one of the main topics for many garden rose breeders (Gudin, 2003). The use of roses in landscaping and public green follows this tendency. Since 2004 the use of pesticides in public green is abolished in Flanders (Belgisch Staatsblad, 2001). Landscape varieties are often chosen for additional characteristics like aesthetical hips in winter time, as a natural fence (Gudin, 1999) or ground cover ... Rose breeding for perfume, medicinal or nutrition industries would not exist because of the lack of breeder’s rights in the countries where these plants are growing (Gudin, 1999).

1.2 Rose breeding

1.2.1 History of rose breeding

The introduction of rose species in the Western world since antiquity and intensive rose breeding since the eighteenth century are common knowledge (Gudin, 1999). However, rose cross breeding is probably much more ancient in the oriental world. There is evidence that roses were already cultivated 5000 years ago by civilizations in China, western Asia and northern Africa. These first acts of domestication and multiplication of species found in the wild led to the spontaneous occurrence of interspecific hybrids that have long been considered as original species. Together with the huge popularity of rose gardens, rose breeding is a feature of the nineteenth and twentieth century. The first records of aimed crosses date back to the beginning of the nineteenth century (Gudin, 2003). Roses are considered to be the first non-edible species in plant breeding. Up to now, rose breeding practices have not changed dramatically since the bursting of this activity (Gudin, 2001).

World-wide there are 25 to 30 highly competitive rose breeding companies and many more amateur breeders (Gudin, 2003). Rose breeding research is carried out by highly competitive private companies, who keep their applied genetic knowledge...
proprietary and unpublished (de Vries & Dubois, 1996). Some companies have established associated research programmes with research groups to improve their methodology. The most applied part of the work is not published, like research on mutation induction, fragrance, pigment or thornlessness heredity (Gudin & Mouchotte, 1996). Notwithstanding pot, cut or garden roses are cultivated in completely different growing systems, (mostly) by different growers, breeders are less specialised in a specific type. This results in “cross-pollination” between the different production types.

Rose breeding has to cope with the difficulty of a high level of heterozygosity, differences in ploidy levels and known problems with sexual reproduction from pollination to seed germination (Gudin, 1999). Although rose chromosomes are small and difficult to observe through cytology, since 1920, studies with historical importance were published by Täckholm (1922) and Hurst (1925 & 1927). Since the 1960’s, rose breeding has benefited from the general gathered knowledge, mainly concerning the sexual reproduction of the species (Gudin, 2001). Little is really known (or published?) on the genetic control of morphological or physiological characters of roses (Gudin, 1999). Some published knowledge on recurrent flowering (Semeniuk, 1971; de Vries & Dubois, 1978), pigmentation (de Vries et al., 1974; de Vries & Dubois, 1978; de Vries et al., 1980; Marshall et al., 1983), winter hardiness (Svejda, 1979) and dwarfness (Dubois & de Vries, 1987) is available. Arene et al. (1993) suggested a close link between genes controlling petal numbers, petal colours and dwarfness. Basic knowledge concerning pollen, pollination, seed maturation and germination (Gudin & Mouchotte, 1996) and the use of amphidiploids (Svejda, 1977) for a better control of hybridisation was published.

Furthermore, selection procedures corresponding to new objectives, such as low temperature and disease tolerance or increased shelf life, have been used (Gudin, 2001). Selection procedures have been described for early prediction of flower productivity and in vitro tests on disease resistance. However, despite some recently acquired genetic data, rose breeding still is very dependent on breeders’ experience, where aesthetical traits based on subjective selection are essential (Gudin, 2003).

For cut flowers most important objectives in breeding are linked with ornamental and quality values like attractive flower colours, tough petals and double flowers, quality of stems, the size of the flowers, vase life and transport qualities and last but not least production capacity. Fragrance is linked with softer petals and a shorter vase-life and has therefore almost completely disappeared in cut roses (Chaanin, 2003). For pot roses inheritance of dwarfness is controlled by a single dominant gene (Dubois & de Vries, 1987). Selection criteria are number of flowers per stem, flower colour, flower size, number of petals and the plant habitus. Other characteristics are linked with production and shelf-life (de Vries, 2003). Disease resistance is the main objective in breeding of garden roses. Despite this, aesthetical characteristics are insuperable. The demand for fragrance is often problematic since some fragrances would be linked with disease susceptibility (Gudin, 1995 & 2003). More and more roses are used in landscaping, therefore ‘carefree’ types are demanded, which means pruning and crop protection measures are not necessary. Besides this the ideal rose has an all-season decorative effect like aesthetic hips during winter (Gudin, 2003).
1.2.2 Biotechnology in rose breeding

Most biotechnological techniques used and developed on other plants were tried on roses, often with limited use in practical breeding work, although some could lead to valuable genotypes. Because of the need for skilled labour and specialised equipment, and also the relatively long time taken to establish them, biotechnological methods are not widespread in the practise of rose breeding (Chaanan, 2003).

Success with ‘incompatible crosses’ can be important to achieve certain goals, especially when making use of interspecific hybridisation. Embryo rescue is often used with success in roses on e.g. *R. rugosa* x *R. foetida* seeds (Drewes-Alvarez, 2003b). Embryo rescue can be done on mature achenes or on immature embryos (Gudin, 1994; Marchant et al., 1994).

Problems in interspecies crosses often arise from ploidy differences. Techniques can be used to overcome these interploidy barriers by reducing ploidy levels through haploidization or to increase ploidy with mitotic, meiotic and somatic polyplody. Mitotic polyploids by use of spindle inhibitors like colchicine and oryzalin (Kermani, 2001) or trifluralin (Zlesak et al., 2005), meiotic polyploidy through unreduced gametes (Crespel & Gudin, 2003) and somatic polyploids by protoplast fusion (Mottley et al., 1996) were obtained in roses. Amphiploid plants have been developed and studied in roses (El Mokadem et al., 2002a&b), the best known amphidiploid is *R. x kordesii*, developed out of *R. rugosa* and *R. wichuraiana*, which gave rise to a group of *R. x kordesii* hybrids. For black spot resistance Baye’s amphiploids 67-305, 86-3 and 86-7 are used in hybridization (Byrne et al., 1996; Byrne & Crane, 2003). Recently, methods of working with and the possibilities of non reduced gametes in diploid and tetraploid roses were described (El Mokadem et al., 2000 & 2002a&b; Crespel & Gudin, 2003). Specific unbalanced meiosis in pentaploid roses of the section Canina is well studied (Nyborg et al., 2000).

Roses are recalcitrant in in vitro work for regeneration of shoots, somatic and gametic embryogenesis (Rout et al., 1999). Only embryogenic cultures have been successfully used to generate transgenic roses. Success rates of somatic embryogenesis, necessary for transformation in roses, are highly cultivar dependent (Kim et al., 2003a). To establish efficient in vitro regeneration systems, plantlets have been regenerated from somatic embryos originating from leaves (de Wit et al., 1990; Rout et al., 1991; Hsia & Korban, 1996; Yokoya et al., 1996; Kintzios et al., 2000; Li et al., 2002a; Kim et al., 2003a), filaments (Noriéga & Sondahl, 1991), petals (Murali et al., 1996), zygotic embryos (Kunitake et al., 1993; Kim et al., 2003b) and root cultures (van der Salm et al., 1998; Kamo et al., 2005). Organogenesis through a callus stage has been achieved from internodes (Lloyd et al., 1988; Ishioka & Tanimoto, 1990; Hsia & Korban, 1996) and from immature embryos (Burger et al., 1990). Protoplasts obtained from embryogenic cell suspensions can be regenerated to plants (Matthews et al., 1991 & 1994; Schum et al., 2001). Direct adventitious shoots have been regenerated from leaves and petioles (Lloyd et al., 1988; Dubois & de Vries 1995; Ibrahim, 1999; Dubois et al. 2000; Ibrahim & Debergh, 2001; Pati et al., 2004). Despite their variety, most of these regeneration systems are extremely time-consuming with very low shoot-
regeneration efficiencies. Moreover, they are highly cultivar-specific and are not always easily reproducible.

There is only a limited amount of publications in roses on Agrobacterium-mediated transformation (Firoozabady et al., 1994; van der Salm et al. 1997, 1998; Li et al. 2002b, 2003; Kim et al., 2004) and biolistic particle bombardment (Marchant et al., 1998a). Transformation approaches are often based on the co-cultivation of established callus derived from leaves and filaments (Firoozabady et al., 1994; Souq et al., 1996, Li et al., 2002b). Van der Salm et al. (1997) made use of nodal stem segments as primary explant. Based on their role in inducing root formation, rol genes have been used to specifically improve the rooting ability of stocks, mainly in woody species; van der Salm et al. (1997) introduced rolB and rolABC genes into rose rootstock 'Moneyway'. Application of sense and antisense approaches to roses, using chs, yielded changes in flower color of dark-red cultivars (Firoozabady et al., 1994; Souq et al., 1996). The sense approach yielded transgenes with flowers ranging from red to pink. The antisense approach yielded light red to magenta flowers. White flowers were not obtained in either case. Marchant et al. (1998b) and Li et al. (2003) used genetic transformation to enhance resistance to respectively black spot and powdery mildew. Biolistic gene delivery was used to introduce a rice gene, encoding a basic (Class I), chitinase into embryogenic callus of the blackspot-susceptible rose ‘Glad Tidings’ (Marchant et al., 1998b). To enhance powdery mildew resistance, an antimicrobial protein gene, Ace-AMP1, was introduced into ‘Carefree Beauty’ via Agrobacterium-mediated transformation (Li et al., 2003).

Molecular markers have been used to investigate polymorphisms in and between species and cultivars and for phylogenetic studies (Ben-Meir & Vainstein, 1994; Vainstein & Ben-Meir, 1994; Millan et al., 1996; Moreno et al., 1996; Reynanders-Aloisi & Bollereau, 1996; Debener et al., 1996). Besides marker techniques, sequence analysis is used for phylogeny in roses (Matsumoto et al., 1997, 1998 & 2000). Studies have been carried out for genetic analysis and mapping of a segregating population with RAPD and AFLP markers (Debener & Mattiesch, 1996). An integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers was constructed (Yan et al., 2005).

Plant variety protection is a hot topic in breeding. Rose cultivars are often difficult to distinguish on morphological characteristics only, therefore since molecular markers were available a lot of effort was put on the use of these markers for variety identification in roses, such as RFLP (Hubbard et al., 1992; Rajapakse et al., 1992; Torres et al., 1993; Ballard et al., 1995), RAPD (Cubero et al., 1995; Reynanders-Aloisi & Bollereau, 1996; Gallego & Martinez, 1996; Matsumoto & Fukui 1996) and AFLP (De Riek et al., 1997 & 2001; Zhang et al., 2000). Moreover, by the UPOV-convention of 1991, essential derived varieties are protected by the original plant breeder’s rights UPOV (1991). In roses for example up to 16 protected colour mutants are known from one original cultivar (‘Frisco’) (Dubois, 2003). Therefore it is important to recognise spontaneous and induced variant types of existing varieties, with molecular techniques like AFLP (Debener et al., 2000; De Riek et al., 2001; Vosman et al., 2004).
1.3 Rose diseases

The main fungal pathogens described on roses are: *Diplocarpon rosae* (black spot) and *Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) (powdery mildew), diseases caused by these pathogens are the subject of this work. Also the Oomycete, *Perenospora sparsa* (downy mildew) can cause serious damage on roses. Rose rust (*Phragmidium* spp.), *Botrytis cinerea* (perfect stage: *Botryotinia fuckeliana*) and *Elsinoe rosarum* (anamorph: *Sphaceloma rosarum*) are other fungal pathogens occurring on roses. Especially *E. rosarum* was for the last couple of years visible in increasing amounts.

1.3.1 Black spot - Diplocarpon rosae

**Taxonomy** (Hawksworth et al., 1995)

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<td>Family</td>
<td>Dermateaceae</td>
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<tr>
<td>Genus</td>
<td><em>Marssonina rosae</em> (Lib.) Lind – <em>Diplocarpon rosae</em> Wolf</td>
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Black spot (*Diplocarpon rosae*) is the most important rose disease of outdoor grown roses. The disease was first reported in Sweden in 1815 by Fries but is now seen worldwide as most cultivars are susceptible. The fungal species is specific for roses and approaches obligate parasitism. The name *Marssonina rosae* is used for the imperfect stage, the perfect stage, *Diplocarpon rosae* is rarely seen (Horst, 1983). The fungus can be grown on artificial media, although growth is extremely slow and the virulence declines (Palmer et al., 1966b). The growth does not exceed 20 mm in 4 weeks time. The fungus can be stored at -20°C or -80°C on leaves or as a suspension of conidia with only a minor loss of vitality (Drewes-Alvarez, 2003a).

Characteristic for this hemibiotrophic disease are purple, brown or black leaf spots up to 4 cm in diameter on the adaxial side of leaves. Often these spots show typical feathery, radiate mycelial strands at the borders of the spots. On the rose canes the same spots without the feathery appearance might be formed. Leaf tissue around the spot starts to grow yellow, mostly leading to defoliation (Horst, 1983; Drewes-Alvarez, 2003a).

Small acervuli (50-400 µm) are formed in the leaf spots and can be seen on the surface. The asexually produced conidia protrude from the acervuli as a white, slimy mass. These conidia (15-25 x 5-7 µm) are two-celled and hyaline (Fig. 1.1). The conidia are dispersed by water and start to germinate within a day. Hyphae with appressoria penetrate the leaf and start to form haustoria and a septate monokaryotic mycelium (Horst, 1983). After appressoria enter the plant, hyphae grow subcuticularly and haustoria appear in the epidermal cells (Drewes-Alvarez, 2003a). Once acervuli have been formed, new conidia can start to spread 10 days...
after infection through rupture of the cuticle. At this stage leaf spots are visible; few weeks later leaves start to fall (Horst, 1983). Normally the disease symptoms are first seen on older leaves, later the fungus spreads upwards. Apothecia, although rarely observed, are formed in the fall. Ascospores are airborne and spread in spring. The fungus overwinters in fallen leaves and in acervuli on stems (Fig. 1.2). The fungus first infects the older leaves down in the plant and spreads with rain. Conidia need wet leaves for at least 7 hours to be able to germinate; the optimal temperature is 18°C. Rainy periods favour the development and spreading of the disease that is most severe by the end of summer. New conidia are formed 10 days after infection of a leaf. For mycelium development 21°C is ideal. In glasshouses in the temperate zones black spot is not seen, because leaves are mostly dry (Horst, 1983).

Pathotypes of black spot are described by use of polyconidial (Palmer et al., 1966a) and monoconidial (Debener et al., 1998; Yokoya et al., 2000a) isolates, also morphotypes were examined (Wenefrida & Spencer, 1993). Described morphological differences are size of conidia, colony mycelium growth and colony colour. There is some discussion on the validity of these differences to make a division in morphotypes. There seems to be a correlation between colony colour and conidial sizes. Longer conidia seem to be formed in lighter coloured colonies compared to small conidia formed in dark colonies. Genetical differences were found with RAPD, SSR and AFLP. Preliminary results indicate the existence of two main clusters of strains (Drewes-Alvarez, 2003a).

A resistance gene $Rdr$ 1 based on a gene-for-gene interaction towards black spot is described in roses and used in marker assisted selection (von Malek et al., 2000; Debener et al., 2003). Some authors suggest fungal development on resistant roses can already cease at spore germination (Dodge, 1931; Reddy et al., 1992). It seems that the cuticle plays a role in the capability of the fungus to colonise the plant because young leaves and leaves wounded with a needle (Dodge, 1931) or abraded leaves (Yokoya et al., 2000a) proved to be more susceptible for the pathogen.

![Fig. 1.1 Back spot symptoms on rose leaves (A); black spot conidia (B)](image_url)
Fig. 1.2 Life cycle of *Diplocarpon rosae* on rose; A: overwintering in infected canes and fallen leaves, B: infection of leaves from overwintering mycelium, water-splashed conidia or airborne ascospores, C: spread from infected material during summer by water-splashed conidia (Horst, 1983)

To learn more about genotype-specific forms of resistance and susceptibility of the host, the presence of partial forms of resistance and different resistance mechanisms were studied by macro- and microscopic analyses. In total, eight interaction types are characterized, five representing compatible and three representing incompatible interactions. The incompatible interactions are characterized by the lack of any visible fungal structures beneath the cuticle, single-cell necroses or necroses of larger cell clusters, the latter two types with penetration hyphae and haustoria in epidermal cells (Blechert & Debener, 2005). Besides these, the influence of PR-proteins after infection of roses by black spot is studied. Sixteen proteins, which are absent or present at a low level in the healthy leaves, have been detected in the intercellular fluid extracted from the infected rose leaves 7 days after inoculation with *D. rosae*. Western-blot analysis shows that rose PR proteins are serologically related to tobacco PR proteins. Infection of black spot results not only in strong accumulation of PR-2, PR-3, and PR-5 proteins in the intercellular spaces of rose leaves but also induction of PR-1 protein at a later stage of infection is seen. However, no systemic induction of PR-1 protein is detected in upper newly expanded and uninfected leaves (Suo & Leung, 2002).
Biotechnological techniques like genetic transformation for enhanced black spot resistance (Marchant et al., 1998b) and amphiploids in resistance breeding (Byrne & Crane, 2003; Carlson-Nilsson, 2000) have been worked on (see 1.2.2). Rose species with a known high resistance to black spot are *R. banksiae*, *R. carolina*, *R. laevigata*, *R. multiflora*, *R. rugosa*, *R. roxburghii*, *R. virginiana* and *R. wichuraiana* (Drewes-Alvarez, 2003a).

### 1.3.2 Powdery mildew - *Podosphaera pannosa*

**Taxonomy** (Braun et al., 2002)

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<tr>
<td>Tribe</td>
<td>Cystothecae</td>
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<tr>
<td>Genus</td>
<td><em>Podosphaera pannosa</em> (Wallr.: Fr.) de Bary</td>
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*Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) is the most occurring disease on roses world wide. It attacks both plants grown in open air like garden roses, and greenhouse roses for cut flower production. The fungus has up to recently been identified as *S. pannosa* (Wallr.: Fr.) Lév. For a long time a division by Woronichine 1914 in two varieties was recognized: *S. pannosa* var. *rosae* infecting roses and *S. pannosa* var. *persicae* infecting peach and almond (Horst, 1983). Cook et al. (1997) could not distinguish the anamorphs of *Sphaerotheca* and *Podosphaera* by use of scanning electron microscopy on the conidial surface. They stated that the difference between *Podosphaera* and *Sphaerotheca* is only made by the host. ITS-sequencing (Takamatsu et al., 1998) and the combination of morphological and ITS-data (Saenz & Taylor, 1999) supported the theory of both species being monophyletic. All *Sphaerotheca* species are finally concluded to belong to the *Podosphaera* genus and are renamed after it (Braun & Takamatsu, 2000; Braun et al., 2002).

The fungus is an obligate parasite. First symptoms are often reddish areas on young rose leaves and new expanded tissue. These leaves are frequently twisted and distorted. Mature leaves are less frequently infected. On stems, closed rose buds and even on the flower, fungal growth may occur. Conidia are airborne and start to germinate at an optimal temperature of 21°C and with a high relative humidity of 100%. Free water can interfere with the inoculation (Horst, 1983). The fungus lives epiphytically on the outer surface of the plant in mats of whitish hyphae. The hyphal strands are anchored to the leaf surface by pegs that penetrate epidermal cell walls, form haustoria for the absorption of nutrients from leaf tissue. Vegetative hyphae form conidiophores that grow upright and produce spores in chains (Fig. 1.3), giving the colony its characteristic powdery appearance (Linde & Shishkoff, 2003). Later in the season a ‘pannose mycelium’ might be formed with ascocarps. This generative form is seldom seen (Fig. 1.4) (Horst, 1983).
Fig. 1.3  Powdery mildew infection on a greenhouse rose (A); a chain of rose powdery mildew conidia (B)

Conidia spread with the wind and germinate 2 to 6h after deposition on a rose leaf. After infection a nipple-shaped appressorium is formed at the end of the germ tube. With enzymatic degradation and mechanical penetration of the cuticle and epidermis, a very fine hypha called an infection peg emerges and enlarges in the lumen of the epidermal cell to form the haustorial neck. One day later, mature haustoria are formed and continue to grow with hyphae that spread over the leaf surface (Linde & Shishkoff, 2003).

Fig. 1.4  Life cycle of *Podosphaera pannosa* on rose, A: the fungus overwinters as mycelium or conidia or ascospores produced in fallen leaves, B. new shoots are infected in spring, C. during summer leaves and flowers are infected by airborne conidia (Horst, 1983)
Pathotypes of rose powdery mildew were described by Bender & Coyier (1984) and Linde and Debener (2003). Only Linde and Debener (2003) made use of monoconidial isolates on a set of differential plants. A dominant resistance gene, $Rpp1$, for race-specific resistance to rose powdery mildew has been reported (Linde & Debener, 2003; Linde et al., 2004).

In literature there is no consistency about different degrees of susceptibility between different horticultural rose classes. Some authors report that climbing roses, ramblers and hybrid teas are more susceptible, whereas others name floribunda and polyantha cultivars to be more susceptible compared to hybrid teas. Within the species there is no difference between sections although some species are known for their good resistance. A hypersensitivity reaction is observed in resistant genotypes. The formation of collars that block the fungus and papillae are seen in roses 1h after wounding by an appressorium (Linde & Shishkoff, 2003).

1.3.3 Other diseases

Other pathogens often found on roses are _Phragmidium_ spp., _Elsinoe rosarum_, _Botrytis cinerea_ and _Peronospora sparsa_.

World-wide 10 _Phragmidium_ spp. are known to be pathogenic on roses, other authors name 30 of the more than 60 _Phragmidium_ species; it is believed this is caused by double naming. The 10 species on rose are: _P. mucronatum_ (Persoon: Persoon) Schlectendal, _P. tuberculatum_ J.M. Müller, _P. fusiforme_ Schröter, _P. americanum_, _P. montivagum_ Arthur, _P. rosae-pimpinellifoliae_, _P. rosae-rugosae_ Kasai, _P. rosae-californicae_ Dietel, _P. rosicola_ (Ellis & Everhart) Arthur and _P. speciosum_ (Fries) M.C. Cooke (Wahyuno et al. 2001). Horst (1983) only mentions nine of these species, _P. rosae-rugosae_ is not listed by this author. In Europe four of these species occur: _P. mucronatum_, _P. tuberculatum_, _P. fusiforme_ and _P. rosae-pimpinellifoliae_ (Horst, 1983; Shattock, 2003). Most occurring are _P. mucronatum_ and _P. tuberculatum_. In the USA _P. mucronatum_ is described as most occurring, while in the United Kingdom this is _P. tuberculatum_ (Howden & Jacobs, 1973; Horst, 1983; Shattock, 2003). It is clear that both species are often confused with one another due to shared characteristics (Shattock, 2003).

Practically no literature is available on _Elsinoe rosarum_. This pathogen is also called _Sphaceloma rosarum_ by name of the anamorphous form, although it is not certain that a generative form was ever observed. Other species of the fungus are reported on _Rubus, Salix_, poinsettia, grapes, cassava, citrus ... The amount of information found on roses does not reflect the presence of the disease in recent years. Last years the fungus appeared in several European countries in an abundant way (Nybom & Debener, personal communication). First reports of the disease date back to 1898. It was even intercepted by plant quarantine inspectors in a bouquet of roses on the first flight of the German dirigible ‘Graf Zeppelin’ in 1928. A reason why this disease is neglected and underestimated is that at first sight symptoms are similar to black spot. During history it is also being confused with _Cercospora_ leaf spot and _Septoria_. Microscopic evaluation of fungal spores can reveal _Alternaria_ as a secondary parasite in the leaf spots (Jenkins, 1932).
Typically, *Elsinoe rosarum* leaf spots are smaller than for black spot and are up to 0.5 cm in diameter. Young spots of this anthracnose disease are red to brown; the centre of the leaf spot turns ash white to a papery membrane that can fall out completely producing a shot-hole symptom (Jenkins, 1932; Horst, 1983). Black spot and *E. rosarum* can both be present in the same leaf spot (Leus, not published).

*Botrytis cinerea* or grey mould (perfect stage: *Botryotinia fuckeliana*) on roses is well documented as this pathogen is also common on various other plant families. *B. cinerea* appears on garden and glasshouse roses, often on the flower itself under humid conditions. This can become a problem during storage or shipment of the flowers. Lesions may appear on petals or on the base of the bud. Canes can be infected showing symptoms on wounds of pruning. Affected plants are often covered with grayish brown mycelium and airborne conidia. Besides conidia in bunches of grapelike structures, sclerotia can be formed for overwintering (Horst, 1983; Gleason & Helland, 2003).

*Peronospora sparsa* or downy mildew is an Oomycete (kingdom Chromista). The disease mostly occurs on young plant parts: leaves, stems, petals ... in purplish-red to dark-brown irregular spots. Often the edges of the leaf spots are clearly defined. The fungus likes a cool and humid climate and will appear only under favourable conditions. Gray conidiophores and conidia can be seen on the lower leaf surface under these conditions, in other cases it is 'sparse' and difficult to detect. Typical are dichotomously branched sporangiophores with pointed sporangia. This disease can cause serious defoliation. Often the disease is confused with black spot (Horst, 1983; Xu and Pettitt, 2003).

### 1.4 Disease resistance breeding

#### 1.4.1 Introduction

Resistance to fungal diseases can have different meanings. Often there is confusion about the use of the words resistance, immunity and tolerance. Resistance is here used in a general sense without detailing the type of resistance. Immunity can be active or passive. An example of immunity through active resistance is the hypersensitive reaction. Immunity through passive resistance is for example a thick wax layer that prevents penetration of the fungus. In tolerant plants the disease is present, although suppressed to some extension.

Plants have developed mechanisms to successfully co-exist in the presence of pathogenic organisms. Some interactions between plants and pathogens are based on recognition of specific elicitor molecules from avirulent pathogen races (*avr* gene products), which is described in the gene-for-gene resistance theory (Tuzun, 2001). In a lot of cases, when spoken of resistance as active immunity, it is based on this gene-for-gene hypothesis of Flor (1955). Since the postulation of resistance genes based on this hypothesis, a lot of effort in breeding research was directed towards this monogenic or so called qualitative or vertical resistance. Other types of resistances, multigenic (also called polygenic, horizontal, partial or quantitative)
resistances, are less well-studied and depend upon multiple genes in the host plant (Tuzun, 2001).

Advances in biotechnology made that over the past 10 years many novel genes, proteins and molecules have been discovered as a result of investigating plant-pathogen interactions. Most attempts to apply this knowledge to engineer improved disease resistance in crops have failed. Although gene efficacy in transgenic plants has often been good, commercial exploitation has not been possible because of the detrimental effects on plant growth, development and crop yield. Moreover, in Europe the public debate on transgenic plants is still not in favour of the technique. Biotechnological approaches have now shifted emphasis towards marker-assisted breeding and the construction of vectors containing highly regulated transgenes that confer resistance in several distinct ways (Hammond-Kosack & Parker, 2003). Molecular approaches will involve the detailed characterization of the many genes that confer resistance, as well as technologies for the precise manipulation and deployment of resistance genes. This will have a significant impact on efforts to ameliorate plant diseases by increasing the definition of and access to gene pools available for crop improvement. Horizontal resistance can be a combination of major and minor disease resistance genes. Mostly this resistance is partial and is often called tolerance. Recently, by further developments in the use of molecular markers for plant breeding, polygenic traits came into interest and are identified by Quantitative Trait Locus (QTL) mapping (Michelmore, 2003). Marker-trait linked polygenic powdery mildew resistance has been studied in tetraploid rose (Yan, 2005). Also in a diploid segregating population QTLs for powdery mildew resistance have been mapped (Dugo et al., 2005).

To be able to create plants resistant to a certain pathogen a lot of information on this pathogen is needed. Most important to the effectiveness and durability of host resistance is the pathogen variability. Pathogen genotypes can interact with specific host genotypes leading to the "breakdown" of resistance within very short periods of time (Brown, 1995). Detection of pathogen variation has traditionally relied upon the identification of virulence variation (races or pathotypes) in the pathogen population by inoculating a sample of pathogen isolates on a series of hosts with defined resistance genes (differentials) and observing the resulting compatible or incompatible disease phenotype. This approach to monitor pathogen populations has been tremendously valuable in the development and deployment of host resistance (Roelfs, 1985; Wolfe & Limpert, 1987; Andrivon & De Vallavieille-Pope, 1993), and has provided important insights into the evolution of pathogen populations in response to selection by host resistance genes relevant to breeding programmes (Kolmer, 1989; Andrivon & De Vallavieille-Pope, 1993).

Virulence variation in plant pathogens were almost always determined in terms of virulence phenotype rather than genotype, which means that frequencies of virulence genes could not be estimated from these assays (Kolmer, 1992). Now, also genomic studies on pathogens have started to provide an understanding of the molecular basis of specificity and the opportunity to select targets for more durable resistance (Michelmore, 2003).
Molecular techniques are often used to describe variation in plant pathogens: RFLP, RAPD and AFLP (Majer, et al., 1996) or DNA sequences. The increased use of molecular biological techniques in plant pathology in the last years has led to a profusion of papers in mycological and plant pathological journals that present data on genetic variation in plant pathogens. The determination of genetic variation in a plant pathogen does not necessarily lead to an increased understanding of pathogen biology. Closer integration of plant pathology and plant breeding programmes will result in population genetic data that are useful to breeders. Closer collaboration between breeders and pathologists and an increased focus on populations of pathogens is required to obtain a better understanding of pathogen variation relevant to breeding efforts.

Information on genetic variation in the pathogens is very important for breeding programmes. Pathogen populations are often geographically sub-structured, which can only be revealed through extensive sampling and the application of appropriate genetic markers. The effectiveness and durability of host resistance can be predicted with a thorough knowledge of pathogen population structure. Screening of resistant germplasm often occurs on one location only (ie. a screening nursery) and/or plants are often inoculated with only a limited number of pathogen genotypes. It is essential to know if the pathogen population at the screening site is representative of variation in the pathogen population once the resistant plants are deployed. For controlled inoculation studies, it is important to expose resistant plants to all potential variation in the pathogen population. This may involve inoculating a much larger number of pathogen genotypes than is currently used in many breeding programmes. The composition of pathogen populations may change through time, which is an important consideration for breeding programmes. The complete replacement of one dominant genotype by another has occurred recently with late blight (Phytophthora infestans on potato and tomato). This kind of changes must be taken into consideration in designing resistance screening programmes (Fry & Goodwin, 1997). Pathogen populations should be monitored on a regular basis to determine if new genotypes have been introduced into a region and whether frequencies of certain pathogen genotypes change over time. The existence of pathogen genotype by host-genotype interactions can have a profound impact on the rate at which pathogens evolve increased virulence on host plants and on the durability of resistance. Resistance that is specific for particular pathogen genotypes is termed race-specific resistance. Resistance which is effective against a large number of pathogen genotypes (ie. lack of interactions) is known as non-race specific resistance or partial resistance (Michelmore, 2000). There are, however, several biological and societal issues that will have to be resolved before the full impact of genomics on breeding for disease resistance is realized (Michelmore, 2003).

On the plant side one of the latest tools in the search for interesting resistances is the Nucleotide Binding-site (NBS) profiling technique based on the use of conserved domains within functionally important families of NBS-containing resistance gene analogues (RGAs) (van der Linden et al., 2004). NBS-profiling has been used to obtain RGAs in R. roxburghii. These were used to map markers for powdery mildew resistance (Xu et al., 2005).
1.4.2 Fungal disease resistance breeding in roses

In contrast to e.g. breeders of vegetables or agricultural crops, where resistance breeding is daily routine, disease resistance has remained an unpopular subject among rose breeders (de Vries, 2000). But, due to changing plant protection legislation and the ecological awareness of consumers, the need to breed rose types with at least a better horizontal resistance is a prerequisite to withstand customer rejection and to regain the economic basis to breed and produce roses (Noack, 2003). In the past, commercial rose breeders have given priority to characteristics of external plant quality like plant habit, flower colour, flower size, flower shape, thorniness or shoot yield. This means that little attention was paid to internal quality including disease resistance. Even more, until recently in the breeding process fungicides were used to protect candidate varieties (de Vries, 2000). An easy systematic combination of traits like in diploids can not be expected in tetraploid rose cultivars; on the other hand it is known that single genes may control disease resistance in roses (Noack, 2003).

Selection for disease resistance in roses can be performed either under field or under laboratory conditions. For an efficient selection under lab conditions the following prerequisites have to be fulfilled (Noack, 2003):

- isolates of the major pathogenic organisms must be available;
- infection assays for major pathogens and pests that make it possible to screen a very large number of seedlings have to be manageable within small and medium-sized companies.

Interesting fungal resistance is mostly found in rose species. The (re)use of these rose species, to extend the germplasm of rose cultivars with disease resistance genes, offers high expectations for breeding. Problems with these crosses are often linked to ploidy differences. Most cultivars are tetraploid while ploidy levels in species can vary from diploid (most frequently) to hexaploid. Crosses between diploid and tetraploid roses yield triploids. As in other plant species, triploids are said to have a problematic fertility. Therefore in roses techniques such as chromosome doubling and the creation of amphiploids are used (1.2.2).

Resistance to powdery mildew varies among rose species and cultivars, and is often pathotype specific. Results of natural and artificial infections with this pathogen show that only few cultivars are highly resistant. In species there is no distinction between the sections concerning resistance. *R. agrestis*, *R. glutinosa* and *R. omeiensis* var. *pteracantha* are mentioned as highly resistant (Linde & Shishkoff, 2003).

Most modern cultivars are susceptible to black spot, but several species show resistance viz.: *R. banksiae*, *R. carolina*, *R. laevigata*, *R. multiflora*, *R. rugosa*, *R. roxburghii* and *R. wichuraiana* (Drewes-Alvarez, 2003). Resistances through transformation and other biotechnological techniques are discussed in 1.2.2.
1.5 Induced resistance

1.5.1 Introduction

Plant defence mechanisms are intensively studied since some years. For responses to fungal pathogens these reactions include the hypersensitivity response, deposition of cell wall strengthening materials such as lignin or callose and the accumulation of active oxygen species (Hammerschmidt, 2003). Induced resistance in general deals with plant responses in which the plant’s resistance to a later challenge is increased by a previous challenge. This resistance response may act locally or can be systemically in the plant and is then called systemic acquired resistance (SAR). The aimed pre-treatment of plants with an inducing organism or compound can incite the plant to mount an effective defence response upon subsequent encounters with pathogens, converting what would have been a compatible interaction to an incompatible one; this is known as induced systemic resistance (ISR). Often only resistance induced by certain rhizosphere bacteria such as plant growth promoting rhizobacteria (PGPR) is defined as ISR, other induced resistances are then called SAR (Hammerschmidt, 1999).

At least in some plant species, induced defence mechanisms depend on the timely accumulation of multiple gene products, such as hydrolytic enzymes, peroxidases or other gene products related to plant defences. ISR induces constitutive accumulation of these and other gene products prior to challenge (Tuzun, 2001). Mechanisms operating locally can be based on structural changes like the formation of papillae, tyloses and abscission zones. Necrosis is possible by oxidative destruction of the cell by hydrogen peroxides and reactive oxygen species. Toxic changes happen by the accumulation of phytoalexins and the synthesis of phenolic compounds and their oxidation. Local defences often lead to the death of the infected cell and a hypersensitivity reaction (HR). Systemic defences broaden the plant’s reaction to parts of the plant distant from the site of infection. This reaction is linked to pathogenesis related proteins (PR), defensins, proteinase inhibitors and cell wall components. Often is referred to the immune system in mammals, but with the difference that in plants ISR is known to function against multiple organisms, and there is no specificity observed in the accumulation patterns of defense-related gene products when ISR is induced.

The timing of the defence responses is critical and can be the difference between being able to cope or succumbing to the challenge of a pathogen or parasite. Great strides have been made over the past 20 years in understanding the physiological and biochemical basis of SAR and ISR. Much of this knowledge is due to the identification of a number of chemical and biological elicitors, some of which are commercially available for use in conventional agriculture. However, the effectiveness of these elicitors to induce SAR and ISR as a practical means to control various plant diseases is just being realized (Vallad & Goodman, 2004). Chemical components with ISR properties are benzo(1,2,3)-thiadiazole-7-carbothiolic acid or BTH known under the trademark Bion and 2,6-dichloroisonicotinic acid or briefly INA. These compounds seem to act similar as salicylic acid (Hamerschmidt, 1999). Other
involved phytohormones are jasmonic acid and ethylene. Plant defence responses activated by the salicylic dependent pathway depend on the specific host-pathogen system. The use of BTH on tomato induced resistance to Botrytis cinerea but not to Oidium neolycopersici. On tobacco the effect was the opposite, resistance could be induced to Oidium neolycopersici but not to Botrytis cinerea. Therefore studies on one system can not be extrapolated to another even closely related system (Achuo et al., 2004).

Rhizobacteria are present in large numbers on the root surface; amongst these, selected strains of beneficial PGPR trigger a plant-mediated ISR response that is effective against a broad spectrum of plant pathogens. Well known bacteria with these characteristics are certain strains of Pseudomonas aeruginosa and P. fluorescens (Pieterse et al., 2003).

1.5.2 Induced resistance in roses

For powdery mildew and black spot, resistance can be induced by use of chemical compounds (Hijwegen et al., 1996; Suo & Leung, 2001). For black spot on roses the effect of BTH was tested by Suo & Leung (2001). They state that the pathogenesis related (PR) proteins β-1,3-glucanase and chitinase had an enhanced activity in the intercellular fluid of in vitro rose leaves after treatment with BTH or infection with black spot. When BTH was applied before inoculation with Diplorcarpon rosae, a diminished disease infection was observed. More PR proteins were described after infection by Diplorcarpon rosae, without applied resistance inducers, but there was no acquired systemic resistance observed in newly uninfected leaves (Suo & Leung, 2002). Hijwegen et al. (1996) used INA to induce resistance against powdery mildew. These authors assume differences between susceptible cultivars and cultivars with partial resistance; as was seen with induced resistance to powdery mildew in cucumber (Hijwegen & Verhaar, 1995) and in barley (Martinelli et al., 1993).
Chapter 2

DIVERSITY IN ROSE PATHOGENS

Rose breeding towards genotypes with an approved resistance against black spot and powdery mildew requires knowledge on the pathogens themselves. In this chapter the occurrence of pathotypes of black spot and powdery mildew is discussed. For both diseases lab cultures were established. The existence of pathotypes was examined by differential reactions on rose genotypes. For powdery mildew virulence on *Prunus avium* is discussed as well, together with the analysis of the ITS-sequence of the pathogen.

This chapter has been partly redrafted after:


2.1 Introduction

The development of a reliable bioassay for screening of parent plants and seedlings is essential to increase the selection efficiency for more resistant rose cultivars by crossbreeding and to establish phenotypic data for molecular research. Hereby not only the genotype of the host plant plays an important role in the host-pathogen interaction, but also knowledge on the variation on the side of the pathogen is required. In this study monoconidial isolates are used to gain knowledge on the occurrence of pathotypes of black spot and powdery mildew.

Concerning variation in plant pathogens two remarks should be made. The first is best expressed in a quote from F.C. Deighton, a specialist on cercosporoid fungi: “If a sparrow flies to a cherry tree, it’s a cherry tree sparrow. If the same sparrow sits in an apple tree, its an apple tree sparrow”. This reflects the species concept often used in the past to name phytopathogens. For example powdery mildews are frequently identified by the host plant (Cook et al., 1997; Yarwood, 1978). Notwithstanding that many plants can become infected by more than one powdery mildew species (Boesewinkel, 1980). Cross inoculations in the past, and DNA sequences today prove that inter- and intraspecies variation is more complex. DNA sequence data have become the standard for species delimitation and population genetics (Kohn, 2004). As a second remark, in contrast, it becomes more and more clear that identification and delineating of individuals and populations is often difficult by population genetic theories (Anderson & Kohn, 1998). Therefore at an interspecies level in a lot of cases pathogens should be considered as species complexes with different possible relations like sibling pathogens, subspecies, varieties and formae speciales (Gudelj et al., 2004). These authors grouped sibling species into three groups based on their host ranges, viz. species that are host specific, species that have partially overlapping host ranges and thirdly, species that have completely overlapping host ranges.

Braun et al. (2002) studied the species concept in powdery mildew fungi. As a base for differentiation purposes of species they use: symptoms on host; morphology and biometry of anamorphs (mycelium, appressoria, conidiophores, conidia, germination) and telemorphs (ascomata, appendages, asci, ascospores); biological specialisation, distribution. The general species concept should be based on morphology; therefore, species should be characterised and distinguished by morphological features, whereas morphologically indistinguishable biological races should be classified as formae speciales. ‘Intermediate’ taxa, which are biologically specialised, but only slightly distinguished in morphology, may be classified as varieties.

In recent years, as in other organisms, plant pathologists studied genetic variation in pathogen populations by use of molecular markers like RFLP, RAPD, AFLP or sequences with a phylogenetic value. The determination of genetic variation in plant pathogens does not necessarily lead to an increased understanding of pathogen biology. However, a closer integration of plant pathology and plant breeding programmes will result in population genetic data useful to breeders.
In breeding, knowledge on pathogen variation is important to control the effectiveness and durability of host resistance. Firstly, pathogen genotypes can interact with specific host genotypes leading to the loss of resistance within very short periods of time (Brown, 1995). Therefore specific pathogen-plant interactions should be studied. Changes of the composition of pathogen populations in time should be taken into account. An example is the change of a dominant genotype by another in late blight (Phytophthora infestans) on potato and tomato. Race specific interactions should be evaluated in another way compared to non-race specific or partial resistance which is thought to be more durable. Secondly, the geographical distribution of pathogen genotypes is an important factor in resistance screening in breeding programmes. Pathogen populations are often geographically substructured. Thirdly, screenings in breeding programmes often occur on one location or with a limited number of pathogen genotypes. In an early screening it is difficult to expose the plant genotypes to a broad variation of the pathogen. The pathogen population at the screening site should be representative for the variation in the pathogen population once the resistant plants are commercially used (Fry & Goodwin, 1997).

The word pathotype is used here to describe the interspecies (racial) variation of a pathogen. Pathotypes can cause differential reactions in a set of plants; it is this knowledge on differential reactions towards fungal isolates that can discriminate pathotypes by testing them on different plant genotypes. On the other hand, pathotypes of a fungus can cause different reactions on plant species and cultivars with different levels of resistance. Different pathotypes can thereby influence a screening towards resistant plants. Although the obvious value of pathotype data, there are limitations to the use of them. For use of information in practice, sets of differentials should be applicable in different labs. Often assays are subject to environmental variation and therefore are difficult to transmit.

2.2 Black spot on roses: pathotype screening

2.2.1 Introduction

For black spot it has been shown that different pathotypes exist on rose species and cultivars (Debener et al., 1998; Yokoya et al., 2000a). Debener et al. (1998) used detached leaves of a differential set of rose genotypes and inoculated these with monoconidial black spot isolates to look for different pathotypes. Yokoya et al. (2000a) did the same on leaf discs. Our study tested monoconidial cultures of black spot collected in Belgium for the occurrence of different pathotypes by a screening on a set of rose genotypes in vitro.

To establish cultures and to gain more knowledge on the practical use of the fungus, preliminary tests were performed. Different media were tested to culture black spot, while the preservation of the cultures was tested at low temperature. The outcome of these tests was used to set up the protocol to obtain monoconidial isolates and to perform a pathotype test on monoconidial isolates.
2.2.2 In vitro culture of Diplocarpon rosae

2.2.2.1 Material and methods

The growth of black spot isolates was tested on 14 different media. On each medium, a colony of 2 mm diameter was transferred. Three different isolates (S-A-2-7; S-A-7-4 and S-A-2-9), collected in Melle – Belgium on rose cultivars, were used in 5 repetitions per medium. These isolates were supposed to be not monoconidial, but were grown out of one leaf spot after disinfection of leaf punches (0.7 mm) in ethanol 70% (10 min) and bleach 1% (1 min). The culture was initiated on potato dextrose agar (PDA) for four weeks.

Media tested for the growth of black spot:
Fungal media:
- PDA: potato dextrose agar (Oxoid 39 g/l);
- MEA: malt extract agar (Oxoid 50 g/l);
- MEB: malt extract broth (Oxoid 20 g/l);
Plant media:
- MS rose medium: Murashige & Skoog (MS) based medium (Murashige & Skoog, 1962): 1 MS micro- & macro-elements including vitamins (Duchefa), 30g/l sucrose, 7g Lab M plant tissue culture agar, 0.05mg/l α-napthalene acetic acid (NAA) and 1mg/l 6-benzylaminopurine (BAP), pH 5.8;
- SH: Schenk & Hildebrandt based medium (Schenk & Hildebrandt, 1972): SH micro and macro elements and SH vitamin mixture (Duchefa), sucrose 30 g/l, 7 g/l agar, 3 mg/l 2,4-D, pH 5.8;
- MS1b: Modified Murashige and Skoog medium: MS-medium micro and ½ macro elements including vitamins (Duchefa) (MS1B), 30 g/l sucrose, 7 g/l agar, 1.5 mg/l thidiazuron (TDZ), 0.5 mg/l indolebutyric acid (IBA), pH 5.8;
Media with rose material:
- PDA with dried rose leaf (80 g/l);
- MEA with dried rose leaf (80 g/l);
- PDA with rose cell suspension (50 ml/l);
- MEA with rose cell suspension (50 ml/l);
- MS with rose cell suspension (50 ml/l);
- SH with rose cell suspension (50 ml/l);
- MS1b with rose cell suspension (50 ml/l);
- MS with an intact rose leaf.

For the medium with dry rose leaf material, healthy leaves of ‘Païline’ were dried at 80°C overnight and ground before mixing in the medium. Rose cell suspensions were obtained by putting rose callus, grown on a leaf of ‘Red Velvet’, in a liquid rose MS-medium in an Erlenmeyer. This cell suspension was shaken for 1 week (100 rpm). Afterwards, the medium was renewed and the culture was used 5 days later. The use of this cell suspension is based on the results of Walker et al. (1996) who had positive effects when cell suspension cultures of roses, Prunus or Rubus were used in media for black spot. For the test with an intact rose leaf, a leaf of ‘Païline’ was disinfected with ethanol 70% for 15 min and bleach 1% for 5 min. The leaf was rinsed twice in sterile water and put on MS-medium in a Petri dish.
Evaluation of the media was performed by:
- Diameter growth: measurement of the diameter of the colony weekly during 4 weeks, with a vernier calliper. Diameters were measured in two perpendicular directions;
- Morphological evaluation: morphological evaluation of the colour of the colony and shape of the conidia formed;
- Germination capacity: testing of the capability of the conidia to germinate on water agar (1%) by plating the conidia in on a Petri dish. Counts of germinated conidia were made 48h later;
- Virulence on in vitro plants: to control the virulence of the conidia, in vitro rose plants in 4 Weck pots (6 plants/pot) were inoculated with 3 ml/pot of 2.10^5 conidia/ml. The test was performed on the cultivars: ‘Red Velvet’, ‘Schneewittchen’, ‘Païline’ and ‘Dream’ for isolate S-A-2-4, and on ‘Kanegem’ and ‘Frisco’ for S-A-7-4. Infection was scored 15 days after inoculation on a 0 to 4 scale (score 0: no infection visible; score 1: one plantlet with a leafspot; score 2: leafspots on 2 or 3 plants; score 3: most plants show leaf spots; score 4: abundant infection on every plant);
- Virulence on detached leaves: detached leaves of ‘Ville du Roeulx’ were inoculated with 2.10^5 conidia/ml of the isolates S-A-2-4 and S-A-7-4. On every leaflet two drops of a conidial suspension were placed. Three repetitions were made with 8 to 26 leaflets. The leaves were evaluated after 15 days. For each repetition the percentage of infected leaflets was calculated.

2.2.2.2 Results

Diameter growth
On none of the media with dried rose leaves, growth of black spot was observed. Upon the media MS, MS with rose cell suspension and SH, the growth of the fungus was significantly higher (Duncan, p<0.05) than for the other media in the first two weeks after the start of the experiment. After four weeks, the colonies had an average diameter from 50 to 63 mm on all media (Fig. 2.1). A similar result was expected from SHC, but because of contamination in some of the plates the results are unreliable. A positive effect of the cell suspension cultures in the media was not observed. The diameter of the fungal growth on the intact leaf on medium and the MEB (liquid) could not be measured. In both cases the fungus developed well.

Morphological evaluation
The colour of the colonies on the different media was orange to brown or pinkish (Fig. 2.2). The colour of the colonies was not correlated with a medium. On some cultures alterations in colour were observed during the experiment. The conidia formed were microscopically evaluated. On all media, the form of the conidia showed modifications when compared to the form found on ex vitro leaf spots. Only on the medium with the intact rose leaf a majority of normal shaped conidia was seen. On all media two cellular large round shaped conidia were formed, on SH and SHC there were also small malformed conidia whereas on MS and MSC conidia appeared in clusters in chain and star form (Fig. 2.3). A prolonged growth on artificial media (6 months and more) increased these malformations.
Fig. 2.1  Mean relative growth of black spot on artificial media; data are mean values ± SD (n=5)

Fig. 2.2  Different colony colours

Fig. 2.3  Normal shaped conidia (A), conidia in chains (B) and star formed conidia clusters (C)
**Germination capacity**
On water agar (1%) in a Petri dish germinated conidia were observed microscopically 48h after plating. Due to the clustering of a lot of the conidia, counting was impossible. It was observed that also deviant conidia could form germination tubes. Normally germination started from the largest of the two-celled conidia; sometimes from both cells. Mostly, germination starts in the axial direction, branching occurs later. In the aberrant conidia generally different germination tubes were formed out of one conidium (up to 6). These germination tubes branched scattered. Only in the conidia formed on the intact rose leaf on medium, normal germination perpendicular to the sept was observed.

**Virulence on in vitro plants**
The highest score for all repetitions made of conidia was obtained from the intact rose leaf. For other media the results varied but it was remarkable that colonies with a brown colour had a significant higher virulence on in vitro plantlets compared to the more pinkish ones (Fig. 2.4). Some of the media produced conidia which had a very low virulence on the in vitro plants tested.

![Virulence on in vitro plantlets of isolate S-A-2-4 cultured on different media (X-axis); expressed as percentages of scores](image)

**Virulence on detached leaves**
The percentage of infected detached leaves was lower than 20% for most media (Fig. 2.5). No leaf spots were observed on detached leaves inoculated with black spot grown on MEAC or MS1B. Only black spot on MSC growing with a brown colony could infect 35% of the leaves. The conidia formed on the intact leaf on MS-medium could infect almost 80% of the detached leaves. This result is comparable to the virulence of conidia collected on infected plants in the field.
Fig. 2.5 Virulence on detached leaves of isolate S-A-2-4 cultured on different media (X-axis); expressed as the mean percentage infected leaflets (±SD) in 3 repetitions (n=35 to 60)

### 2.2.2.3 Discussion

In vitro culture of black spot isolates was tested on different media. All conidia obtained on artificial media showed a serious decline in virulence. The best overall result was obtained with conidia grown on a rose leaf. Only here the morphology and germination were normal, while there was no loss of virulence when tested on detached leaves. Colony growth on leaves could not be compared to growth on artificial media. Growth on artificial media was extremely slow. Degeneration of the conidia could be observed through changes in conidia shape.

The higher growth rate on some media in the first weeks of the culture could be due to the nutritional richness of these media. Some authors use morphological differences such as colony growth and colour to determine different black spot pathotypes (Drewes-Alvares, 2003a). In our observations as well, some isolates showed differences and alterations in colony colours. Paler colonies gave less virulent conidia.

Different authors discussed the rapid loss of virulence when black spot is grown on artificial media (Knight & Wheeler, 1978b; Walker et al., 1996). Germination of the conidia and growth of the colony on artificial media is very slow as previously mentioned by Horst (1983). Notwithstanding conidia from all media could form germination tubes, virulence on leaves declined. Therefore the time necessary to establish a monoconidial culture on artificial media should be as short as possible to circumvent virulence loss.
2.2.3 Storage of black spot

2.2.3.1 Material and methods

Storage
The effect of low temperatures on conidial virulence was evaluated after two days storage at -20°C of black spot infected leaves. To describe the effect of low temperatures after storage for a longer period, black spot infected leaves were stored 1 year at -20°C and -80°C. Infected leaves were collected in the field.

Inoculation
After thawing the stored leaves, a conidia suspension was made by putting the leaves in water for 1h. Virulence of the conidial suspension was tested by spraying 2.10^4 conidia/ml until 'run off' on 3 cuttings of 4 cultivars ‘Wettra’, ‘Ville du Roeulx’, ‘Gomery’ and ‘Melrose’. The cuttings were 8 months old. Infected plants were put under plastic during 24 h to preserve a high relative humidity. The plants were evaluated 18 days later. All leaves were scored individually from 0 (not infected) to 5 (abundant appearance of leaf spots and yellowing of the leaf). In average, the plants had 19 leaves.

Disease index
Mostly the occurrence of a disease is scored on a certain scale representing the severity of the disease outbreak as e.g. percentage of the leaf infected or number of lesions. While scoring, often the different leaves of a plant are scored individually. To calculate a global score for the plant expressed as a percentage, the disease index (DI%) can be calculated. In this disease index the occurrence of the disease is calculated as a percentage of the worst possible infection by this disease.

\[
DI\% = \frac{\sum_{i=0}^{n} (#\text{leaves with score } i \times i)}{(#\text{ leaves} \times n)} \times 100
\]

with \(i = \) lowest score and \(n = \) highest score possible (Liu et al., 1996)

2.2.3.2 Results

Although there is some variation in the amount of leaf spots, there was no loss of virulence after cold storage (Table 2.1). Only on ‘Melrose’ the conidia that were stored for 1 year showed less leaf spots. Even without storage the infection rate on this cultivar was very low. There was variation within the same treatment, partly because of secondary infections on the leaves what made statistical analyses inappropriate.
**Table 2.1**  Mean DI% on rose cuttings after black spot inoculation with fresh and stored conidia

<table>
<thead>
<tr>
<th>Rose cultivar</th>
<th>Mean DI%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no storage</td>
</tr>
<tr>
<td>'Wettra'</td>
<td>6.4</td>
</tr>
<tr>
<td>'Ville du Roeulx'</td>
<td>17.1</td>
</tr>
<tr>
<td>'Gomery'</td>
<td>3.6</td>
</tr>
<tr>
<td>'Melrose'</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**2.2.3.3 Discussion**

Cold storage of infected leaves without loss of virulence was possible at -20°C and at -80°C. It is advisable to establish a control infection after cold storage on a susceptible cultivar like 'Ville du Roeulx'. Storage at -20°C or -80°C is a good option to preserve black spot isolates for a longer time, compared to cultivation on artificial media which gives a rapid decline in virulence. Also Drewes-Alvarez (2003a) obtained good results with conidia stored at -20°C or -80°C.

**2.2.4 Occurrence of pathotypes of Diplocarpon rosae on roses in Belgium**

**2.2.4.1 Material and methods**

**In vitro rose medium**

In vitro axillary rose cultures were established and maintained on a Murashige & Skoog medium (Murashige & Skoog, 1962): 1 MS micro- & macro-elements including vitamins (Duchefa), 30g/l sucrose, 7g Lab M plant tissue culture agar, 0.05mg/l α-naphtalene acetic acid (NAA) and 1mg/l 6-benzylaminopurine (BAP), pH 5.8. Media were autoclaved (1 bar, 120°C, 20 min). Plants were subcultured every 2 months.

**Establishment of monoconidial isolates**

Fungal development was established in the lab by growing black spot leaf discs on potato dextrose agar (PDA). Hence, the leaves were cleaned a first time in water with detergent. In a laminar flow leaf discs were punched out with a cork borer (0.7 mm). Disinfection of the leaves was done in ethanol 70% (15 min) and bleach 1% (1 min) followed by rinsing two times in sterile water. The discs were dried on filter paper until they were completely dry. On a Petri dish 5 discs were placed. Not earlier than two weeks from the start of the culture a start of fungal growth could be observed. For some isolates it took several months before the first symptoms of development of black spot were visible on the medium. For all isolates, growth on artificial media was very slow (<1 cm²/month). Cultures were obtained by streaking a conidial suspension prepared from the growth of the fungus out of a leaf discs, on potato dextrose agar. In a dilution, single emerging conidia were isolated with a needle and put on a disinfected rose leaf on rose medium in a Petri dish. Established monoconidial cultures were multiplied and maintained on in vitro rose plantlets in test tubes by putting droplets of a conidia suspension in sterile water on the plantlets (Fig. 2.6).
infected leaf material is sampled
↓
wash with detergent
↓
incubation overnight at 22°C to control conidia formation
↓
leaf punches are made (0.7 mm)
↓
store in a plastic bag at –20°C
↓
desinfection of the leaf punches with
15 min 70% ethanol + 5 min 1% bleach
rinse at least 2 times with sterile water
↓
first fungal growth is visible after at least 2
weeks culture of the leaf punches on PDA medium
↓
conidial suspension is plated on MS medium
↓
monoconidial growth is visible after 2 weeks
↓
monoconidial cultures are used to inoculate
a rose leaf on MS medium
↓
in vitro rose plant can be inoculated with the
storage of infected leaves -20°C
monoconidial culture growing on the rose leaf
↓
conidia to be used for inoculation

Fig. 2.6 Scheme for establishment of monoconidial isolates of black spot on in vitro rose plants

**Black spot isolates**
Isolates of black spot were collected on different rose cultivars at 6 locations in Belgium:

- A DvP - Melle
- B nursery Jan Van Herreweghe - Serskamp
- C public green - Smetlede
- D nursery Rudy Velle - Oudenburg
- E nursery Houtmeyers - Laakdal
- F public green – Lesdain

The monoconidial cultures were evaluated on 7 rose cultivars and species: ‘Wettra’, ‘Gomery’, ‘Melrose’, ‘Ville du Roeulx’, ‘Excelsa’ (syn.: ‘Red Dorothy Perkins’), *R. laevigata anemoides* and *R. wichuraiana*. Conidia for inoculation were collected on infected in vitro plantlets and conidial suspensions were prepared (2.10⁴ conidia/ml) by putting infected in vitro leaves in sterile water (1 ml) in an Eppendorf tube that was gently vortexed. For inoculation, freshly transferred plants in test tubes were used.
Every leaf of the test plants (mostly 3 to 4 leaves) was inoculated with 10 µl of conidial suspension. For every cultivar and species at least 15 plants were inoculated, hence over 45 leaves were scored for every combination of monoconidial culture and rose cultivar or species. Each leaf was scored weekly during 4 weeks after inoculation. A score ranging from 0 to 5 was utilized (Fig. 2.7). To score a binocular (64x) was used.

Score 0: no infection visible
Score 1: a (suspected) beginning of infection with a small leaf spot
Score 2: conspicuous black spot leaf spot(s), no visible development of acervuli
Score 3: several leaf spots with yellowing of the leaf and start of formation of acervuli
Score 4: leaf spots on all leaflets and severe development of acervuli.
Score 5: the leaf died

Fig. 2.7  Scores for black spot infection on in vitro rose leaves

Afterwards, a class was given to each combination of rose cultivar/species and monoconidial isolate. This class was calculated according to the highest occurring scores (in week 4), on condition that at least 10% of the scored leaves within the tested rose genotype belonged to this class.

Three classes were used:
- scores 0 and 1: not virulent
± scores 2 and 3: moderately virulent
+ scores 4 and 5: highly virulent
For example if only score 0 and 1 appeared this was called class “-” (not virulent) (less than 10% of the leaves in a higher class). If the highest scores (>10% of the leaves) were 2 or 3 this is called class “±” (moderately virulent). In case at least 10% of the leaves scored 4 or 5 the class is “+” (highly virulent).

2.2.4.2 Results

In table 2.2 the classes attributed to the combination between the monoconidial culture and test species or cultivar are summarised. From these results it is clear that differential reactions appear between different black spot isolates on some of the rose species and cultivars. Isolates B and D together with E and F show similar virulence reactions on the different rose genotypes, whereas A and C are different from the other isolates tested. These differential reactions point out the different pathotypes. Figure 2.8 shows the distribution of the classes of resistant, intermediate and susceptible reactions as a percentage of the tested leaves in each combination. By this, individual combinations can be compared. For example the infection on ‘Melrose’ shows for all tested isolates a low virulence. On ‘Ville du Roeulx’ isolate A can not establish infection, isolates B, C and D are moderately virulent, while this cultivar seems to be highly susceptible to isolates F and E.

2.2.4.3 Discussion

Four different pathotypes could be found in the six isolates collected in Belgium. The differences found indicate a gradation from less to more virulent. Isolate A was least virulent; monoconidial isolates E and F proved to be the most aggressive.

Differential reactions appeared in the cultivars ‘Gomery’, ‘Wettra’, ‘Excelsa’, but most of all in ‘Ville du Roeulx’ and *R. laevigata anemoides* where all three responses: resistant, intermediate and susceptible reaction to the different monoconidial cultures could be observed. ‘Melrose’ and *R. wichuraiana* did not reveal differential reactions towards the black spot isolates tested. ‘Melrose’ a rose cultivar known for its good overall disease resistance showed an intermediate resistance towards all tested isolates. *R. wichuraiana* was the only rose with excellent resistance to all isolates in this test.

These results confirm earlier research on pathotypes in Germany and Great-Britain, where also a variation in occurring isolates could be shown (Debener et al., 1998; Yokoya et al., 2000a). Disease resistance was only found in the species *R. wichuraiana*; indicating, as generally accepted that the introduction of disease resistance in modern cultivars will have to be established by crosses with resistant wild species.
Table 2.2 Interactions between 6 monoconidial isolates of *Diplocarpon rosae* and 7 rose cultivars and species

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Classes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td><code>Gomery</code></td>
<td>±²</td>
</tr>
<tr>
<td><code>Wettra</code></td>
<td>±</td>
</tr>
<tr>
<td><code>Melrose</code></td>
<td>±</td>
</tr>
<tr>
<td><code>Ville du Roeulx</code></td>
<td>-</td>
</tr>
<tr>
<td><code>Excelsa</code></td>
<td>-</td>
</tr>
<tr>
<td><em>R. laevigata anemoides</em></td>
<td>-</td>
</tr>
<tr>
<td><em>R. wichuraiana</em></td>
<td>-</td>
</tr>
</tbody>
</table>

² `+` good development of black spot; `±` moderate development of black spot; `-` no development of black spot; `/` not tested

Fig. 2.8 Percentage of leaves infected for every combination of rose genotype and monoconidial isolate. Three different classes are used: no, moderate and good development of black spot. Plants tested are: *R. wichuraiana* (Wi), `Melrose` (Me), `Excelsa` (Ex), `Ville du Roeulx` (VD), `Gomery` (Go), `Wettra` (We) and *R. laevigata anemoides* (La)
2.3 Powdery mildew on roses: pathotype screening

2.3.1 Introduction

Except for roses, few data exist on the host range of this pathogen. For a long time, a division by Woronichine made in 1914, into two powdery mildew varieties was recognized: Sphaerotheca pannosa var. rosae infecting roses and S. pannosa var. persicae infecting peach and almond (Horst, 1983). Cook et al. (1997) could not distinguish the anamorphs of Sphaerotheca and Podosphaera by observing conidial surfaces with a scanning electron microscopy. They stated that the difference between Podosphaera and Sphaerotheca is only made by the host plant. Internal transcribe spacer (ITS) data (Takamatsu et al., 1998) and the combination of morphological data with ITS sequencing (Saenz & Taylor, 1999) supported the theory that both genera are congeneric. It was concluded that all Sphaerotheca species belong to the Podosphaera genus. The fungus previously identified as S. pannosa (Wallr. Ex Fr.) Lév. is now called Podosphaera pannosa (Wallr.: Fr.) de Bary (fam. Erysiphaceae, tribe Cystotheceae) (Braun & Takamatsu, 2000; Braun et al., 2002). To investigate the evolutionary history of Erysiphaceae tribe Cystotheceae ITS sequences were investigated by Takamatsu et al. (2000). The authors suggest that the Cystotheceae were originally arbor-parasitic, and transition to herb-parasitism may have occurred on at least two independent occasions. This transition is believed to have occurred in the Rosaceae.

Powdery mildews continue to expand their host range as might be seen in the amount of recent publications announcing first reports on new host plants (Gaetan & Madia, 2004; Garibaldi et al., 2004; Koike & Saenz, 2004; Kurt et al., 2004, Soylu et al., 2004). This complicates accurate identification when identification is linked to host specificity. Although Podosphaera (Sphaerotheca) pannosa is believed to grow only on Rosaceae, its occurrence has also been reported on Eucalyptus spp. The identity was confirmed by ITS sequencing (Cunnington et al., 2003).

Pathotypes of powdery mildew on roses have been described by Mence & Hildebrandt (1966), Bender & Coyier (1984) and by Linde & Debener (2003). In our preliminary research monoconidial isolates of powdery mildew were tested for differential reactions on rose genotypes. Later it was observed that some of the isolates were also virulent on Prunus avium (Leus et al., 2002 & 2003).

The three genera of the tribe Cystotheca contain only one ascus in their cleistothecium. The genera can be distinguished by their cleistothecial morphology, especially the appendage morphology, i.e. dichotomously branched appendages in Podosphaera, mycelioid appendages in Sphaerotheca, and only a few short simple appendages in Cystotheca (Takamatsu et al., 2000). Cook et al. (1997) stated that only the host plant makes a difference in identifying the species after they tried to distinguish anamorphs of Sphaerotheca and Podosphaera by observing conidial surfaces with a scanning electron microscope.
Until recently both genera were believed to be organised as described by Takamatsu et al. (2000). The genus *Podosphaera* has 250 host plants, of which 216 hosts (86.4%) belong to *Rosaceae*, including the genera *Crataegus*, *Malus*, *Prunus*, *Pyrus*, *Sorbus* and *Spiraea*. The remaining 34 hosts are scattered amongst 12 plant families including *Ericaceae*, *Hamamelidaceae*, and *Caprifoliaceae*. The genus *Podosphaera*, therefore, is considered to be concentrically parasitic to *Rosaceae* and sporadically to other plant families. Almost all of the hosts of *Podosphaera* are woody plants, indicating that *Podosphaera* is also arbor-parasitic. On the other hand, *Sphaerotheca* section *Sphaerotheca* has many (806) host plants compared with *Sawadaea*, *Cystotheca*, and *Podosphaera*. Of these, 456 (56.7%) hosts are *Rosaceae* and the remaining 349 hosts are scattered amongst 27 plant families including *Euphorbiaceae*, *Geraniaceae*, *Onagraceae*, *Saxifragaceae* and *Polemoniaceae*. Most of these host plants are shrubs or herbs. *Sphaerotheca* section *Sphaerotheca* is, thus, considered to be shrub- or herb-parasitic. *Sphaerotheca* section *Magnicellulatae* also has many host plants and a wide host range. Out of the total of 1110 host plants, 44.7% are *Asteraceae* and 18.6% are *Scrophulariaceae*. The remaining 408 host plants are scattered amongst 38 plant families including *Cucurbitaceae*, *Fabaceae*, *Brassicaceae*, *Dipsacaceae*, *Ranunculaceae* and *Lamiaceae*. Most of these hosts are herbaceous plants, indicating that *Sphaerotheca* section *Magnicellulatae* is herb-parasitic. It is worth noting that the *Rosaceae* is not included in the host families of the section *Magnicellulatae* (Takamatsu et al., 2000). Takamatsu et al. (2000) stated after studying the ITS-sequences of the *Cystothecaceae* that *Podosphaera* and *Sphaerotheca* do not separate into different clades. But, *Podosphaera* species parasitic to *Prunus* and *Sphaerotheca* section *Magnicellulatae* group together. The remaining *Podosphaera* species and *Sphaerotheca* section *Sphaerotheca* (like *S. pannosa*) form another subclade (Fig. 2.9). In contrast to earlier opinions, *Podosphaera* can be regarded as ancestral to *Sphaerotheca*; the two sections of *Sphaerotheca* are derived from *Podosphaera* on at least two different occasions.

By ITS-data (Takamatsu et al., 1998) and the combination of morphological data with ITS-sequencing (Saenz and Taylor, 1999), all *Sphaerotheca* species were finally concluded to belong to the *Podosphaera* genus and renamed after it (Braun & Takamatsu, 2000; Braun et al., 2002). The former *Sphaerotheca* section *Sphaerotheca* and section *Magnicellulatae* are now *Podosphaera* section *Sphaerotheca* and subsection *Sphaerotheca* and subsection *Magnicellulatae* (Braun et al., 2002).

In this research we examined isolates of powdery mildew collected on rose and *Prunus* spp. by differential plant reactions and ITS-sequencing to provide more insight in the host range of *Podosphaera pannosa*.
Cladogram of the major clades of the tribe Cystotheceae and their host plants. The number within the parentheses shows the number of host plants in that respective plant family. The tree is made after ITS-sequencing by Takamatsu et al. (2000). After renaming Sphaerotheca to Podosphaera; the former Sphaerotheca section Sphaerotheca and section Magnicellulatae are called Podosphaera section Sphaerotheca subsection Sphaerotheca and subsection Magnicellulatae (Braun et al., 2002).
2.3.2 Material and methods

**Powdery mildew isolates**
Powdery mildew isolates used in this study are listed in Table 2.3. They were sampled from outdoor grown roses in Belgium (13 samples), France (2 samples), The Netherlands (1 sample), Germany (4 samples) and Israel (1 sample). Four powdery mildew isolates were sampled from different *Prunus* spp. in Belgium. Monoconidial isolates were established by use of the eyelash technique. One conidium was taken with an eyelash from an infected leaf and placed on the leaf of an in vitro plantlet in a test tube. Afterwards monoconidial isolates were maintained on in vitro rose plantlets, ‘Red Velvet’ and ‘Inka’, or *Prunus avium* plants, respectively. Every 4 to 6 weeks fungal isolates were subcultured on fresh in vitro plantlets by taking conidia with a paint brush.

**In vitro inoculation methods**
Rose plants were cultivated on MS-medium in test tubes (Murashige and Skoog, 1962) (see 2.2.4.1). The in vitro *Prunus avium* plants were grown on MS-medium supplemented with 30 g/l sucrose, 1.5 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l gibberellic acid (GA₃) and 7 g/l Lab M plant tissue culture agar. The pH of the medium was adjusted to 6.2 (Meier-Dinkel, 1986). The media were autoclaved (20 min - 120°C - 1 bar) before use. To test for differential plant reactions, inoculation of in vitro plants was performed with a paint brush. For every isolate – plant genotype combination at least 12 plants were inoculated. Every plant had 3 leaves or more and all leaves were inoculated. Tested plant genotypes were the *R. hybrida* cultivars ‘Melrose’, ‘Gomery’, ‘Ville du Roeulx’, ‘Wettra’ and ‘Excelsa’, *R. wichuraiana* Crépin, *R. laevigata anemoides* Michx. and *Prunus avium* L. The number of conidia used was not quantified, but the presence of conidia was confirmed by microscopic observation.

**Disease index**
Each leaf was scored weekly, up to 4 weeks after inoculation. A score ranging from 0 (no infection) to 5 (leaf died) was used (Fig. 2.10). To be able to detect leaves with score 1, a binocular (64x) was used. Afterwards the disease index (DI%) of each plant was calculated according to Liu et al. (1996) (see 2.2.3.1). The average DI% was calculated only on the infected plants. Plants with DI% 0 were considered as not well infected, except for those cultivars/species where no single plant was infected, there the average DI% was considered to be 0.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no infection visible</td>
</tr>
<tr>
<td>1</td>
<td>a (suspected) beginning of infection with development of hyphae</td>
</tr>
<tr>
<td>2</td>
<td>clearly recognisable infection by powdery mildew with development of the first conidia</td>
</tr>
<tr>
<td>3</td>
<td>several infected leaflets with formation of conidia</td>
</tr>
<tr>
<td>4</td>
<td>abundant formation of hyphae and conidia on all leaflets</td>
</tr>
<tr>
<td>5</td>
<td>the leaf died because it is overgrown with powdery mildew</td>
</tr>
</tbody>
</table>
While scoring it was observed that morphology of the fungus on the different plant cultivars and species could differ. Remarks were made on the development of hyphae and conidia. Some species showed an altered balance between formation of mycelium and spores, therefore the formation of mycelium and spores was evaluated morphologically by use of a binocular (64x).

For rose isolates growing on in vitro Prunus avium plants, inoculations were made on detached leaves to control virulence on Prunus avium ex vitro. Conidia were dusted on leaves placed on wet paper in a Petri dish.

Out of the individual scores and disease indexes, the reactions of the plant towards the different isolates were grouped in three classes: normal development, restricted development of mycelium and/or conidia and no development of the fungus. Inoculations of isolates on plant genotypes not resulting in growth of powdery mildew were repeated to avoid misinterpretation due to poor conidia quality or other inoculation problems. Uninoculated controls never showed powdery mildew symptoms.

![Scores for powdery mildew infection on in vitro rose leaves](image)

**Greenhouse validation test**

Validation of the in vitro test was done on cuttings of ‘Wettra’, ‘Gomery’, ‘Melrose’, ‘Ville du Roeulx’ and ‘Excelsa’ in a greenhouse. Ten week old cuttings were infected with a paint brush using an inoculum collected from infected greenhouse roses. For each cultivar at least 6 plants were used. All leaves were infected and scored separately, four weeks after inoculation. Disease indexes were calculated for each plant.
DNA-extraction

DNA-extraction of powdery mildew were made, based on the protocol of Saenz and Taylor (1999). Conidia were washed from the leaf with a repeated run-off with 700 µl of a 5% Chelex 100 solution. The conidia were collected in an Eppendorf tube and autoclaved (20 min - 120°C - 1 bar). After autoclaving the solution was centrifuged for 10 min (20000g).

Primers

Two forward and one reverse primer were used in two PCR reactions to amplify the ribosomal DNA of the ITS region, with a small portion of the flanking 18S and 28S genes, the complete ITS1 region, the complete 5.8S gene and the complete ITS2 region. The ITS region is widely used to show genetic variability and characterisation at species level (White et al., 1990). For the initial reaction the forward primer ITS1f (5’ CTTGGTCATTTAGGAAGTAA 3’) (Gardes & Bruns, 1993) and reverse ITS4 (5’ TCTCCGCTTATTGATATGC 3’) (White et al., 1990) were used. The forward and reverse primers for the second, semi-nested PCR reaction were respectively ITS5 (5’ GGAAGTAAAAAGTCGTAACAGG 3’) (White et al., 1990) and ITS4.

PCR-reactions

An initial PCR amplification was performed on 5 µl of the Chelex DNA template together with a reaction mixture according to Cunnington et al. (2003), 9.65 µl H2O, 200 µM of each dNTP, 1.5 mM MgCl2, 2.5 µl 10x PCR-buffer (Applied Biosystems), 0.25 µM of each primer ITS1f and ITS4, 100 ng BSA and 0.5 U Amp Taq Gold. The reaction mixture was run for 10 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, 2 min at 72°C, and a final extension of 10 min at 72°C.

A second, semi-nested PCR was performed on 1 µl of PCR product of the initial reaction product together with 0.5 µM of the primers ITS5 and ITS4 in a reaction mixture with the same composition as for the initial PCR, but in a total volume of 50 µl (33.65 µl H2O). Cycling times were the same but the annealing temperature was set to 60°C. The PCR-product was purified with the QIAquick PCR purification kit (Qiagen). To ensure that material was amplified, PCR-product was detected by running 4 µl PCR-product in 2 µl loading buffer on a 1.5% agarose gel in 1X TAE. This detection was done after the nested PCR. The sequencing PCR was performed with the primers ITS5 and ITS4. In the sequencing PCR fluorescent labelled dNTP’s were used. Finally a second purification was done and the sequencing was run on an ABprism 377 (Perkin Elmer Co.).

Data analysis

Sequences were aligned and analysed using BioEdit (Hall, 1999) and Clustal-X (Thompson et al., 1997). Sequences were compared to sequences obtained by Cunnington et al. (2003 and 2005); Saenz & Taylor (1999) and Takamatsu et al. (1998 and 2000) published in the database of NCBI. From a distance matrix, based on the number of base pair differences between isolates, a neighbour-joining tree was constructed using Phylip (Felsenstein, 1993).
Table 2.3  Origin of the powdery mildew isolates sampled on rose and *Prunus avium*

<table>
<thead>
<tr>
<th>Code</th>
<th>Host</th>
<th>Country</th>
<th>Location</th>
<th>Year</th>
<th>GenBank No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Rose isolates</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-C1</td>
<td>unknown cv.</td>
<td>Belgium</td>
<td>Smetlede</td>
<td>2000</td>
<td>DQ139411</td>
<td>Leus et al. 2002</td>
</tr>
<tr>
<td>R-D</td>
<td>‘Pascali’</td>
<td>Belgium</td>
<td>Oudenburg</td>
<td>2000</td>
<td>DQ139430</td>
<td>Leus et al. 2003</td>
</tr>
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<td>unknown cv.</td>
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<td>Lesdain</td>
<td>2000</td>
<td>DQ139414</td>
<td>Leus et al. 2002</td>
</tr>
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<td>Lesdain</td>
<td>2000</td>
<td>DQ139415</td>
<td>Leus et al. 2002</td>
</tr>
<tr>
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<td>Heers</td>
<td>2000</td>
<td>DQ139416</td>
<td>Leus et al. 2002</td>
</tr>
<tr>
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<td>unknown cv.</td>
<td>Belgium</td>
<td>Heers</td>
<td>2000</td>
<td>DQ139417</td>
<td>Leus et al. 2002</td>
</tr>
<tr>
<td>R-V1</td>
<td>‘Bishop Darlington’</td>
<td>Belgium</td>
<td>Oudenburg</td>
<td>2003</td>
<td>DQ139418</td>
<td>This study</td>
</tr>
<tr>
<td>R-V2</td>
<td>‘Mme. G. Staechelin’</td>
<td>Belgium</td>
<td>Oudenburg</td>
<td>2003</td>
<td>DQ139419</td>
<td>This study</td>
</tr>
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<td>unknown cv.</td>
<td>Belgium</td>
<td>Oudenburg</td>
<td>2003</td>
<td>DQ139420</td>
<td>This study</td>
</tr>
<tr>
<td>R-R</td>
<td>‘Rivierenhof’</td>
<td>Belgium</td>
<td>Belsele</td>
<td>2003</td>
<td>DQ139432</td>
<td>This study</td>
</tr>
<tr>
<td>R-P</td>
<td>‘Rivierenhof’</td>
<td>Belgium</td>
<td>Destelbergen</td>
<td>2004</td>
<td>DQ139433</td>
<td>This study</td>
</tr>
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<td>R-M2</td>
<td>unknown cv.</td>
<td>France</td>
<td>Carnet-des-Maures</td>
<td>2003</td>
<td>DQ139421</td>
<td>This study</td>
</tr>
<tr>
<td>R-M3</td>
<td>unknown cv.</td>
<td>France</td>
<td>Carnet-des-Maures</td>
<td>2003</td>
<td>DQ139422</td>
<td>This study</td>
</tr>
<tr>
<td>R-2</td>
<td>‘Caramba’</td>
<td>Germany</td>
<td>Ahrensburg</td>
<td>2000</td>
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<td>Linde and Debener, 2003</td>
</tr>
<tr>
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<td>2000</td>
<td>DQ139425</td>
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</tr>
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<td>Tel Aviv</td>
<td>2000</td>
<td>DQ139424</td>
<td>Linde and Debener, 2003</td>
</tr>
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<td>Arpke</td>
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<td>DQ139426</td>
<td>This study</td>
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<td>unknown cv.</td>
<td>Germany</td>
<td>Siebeldingen</td>
<td>2003</td>
<td>DQ139427</td>
<td>This study</td>
</tr>
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<td>R-N</td>
<td>unknown cv.</td>
<td>The Netherlands</td>
<td>Grootebroek</td>
<td>2003</td>
<td>DQ139431</td>
<td>This study</td>
</tr>
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<td></td>
<td><em>Prunus isolates</em></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P-G</td>
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<td>Belgium</td>
<td>Geraardsbergen</td>
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<td>DQ139432</td>
<td>This study</td>
</tr>
<tr>
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<td><em>P. laurocerasus</em></td>
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<td>Brugge</td>
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<td>DQ139428</td>
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<td>P-M</td>
<td><em>P. laurocerasus</em></td>
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<td>Maldegem</td>
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<td><em>P. lusitanica</em></td>
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<td>Serskamp</td>
<td>2004</td>
<td>DQ139435</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.3.3 Results

The collected powdery mildew isolates were used to infect different rose genotypes and *Prunus avium* (Table 2.4). All isolates collected from roses were highly virulent on the different rose cultivars and weakly virulent on ‘Excelsa’. Isolates F1 and F2 were able to establish a minor infection with very few mycelial strands on *R. wichuraiana*. Eighteen isolates developed well on rose cultivars but not or to a very low extend on *Prunus* (Table 2.4). A group of four isolates (R-D, R-N, R-P and R-R) grew well on both rose cultivars and *Prunus avium*. These isolates were also virulent on detached leaves of ex vitro *Prunus avium* plants. The isolates R-D, R-P and R-R did not infect *R. laevigata*, while R-N was able to grow on both *Prunus avium* and *R. laevigata anemoides*. The isolates collected on *Prunus* spp. could infect *Prunus avium* but did not develop on any of the used rose genotypes (Table 2.4).

Most infections classified as weakly virulent (±) only developed a few hyphae without or with almost no conidia formation. For all infections on ‘Excelsa’ there was only growth of hyphae with a very limited growth of conidia. On *R. laevigata anemoides* there was a restricted growth of mycelium, resulting in a small infection site, but with the formation of a lot of conidia (Table 2.5).

ITS analysis revealed that all isolates collected from roses had the same sequence, except for the four isolates (R-D, R-N, R-R and R-P) able to infect both roses and *Prunus avium* (Fig. 2.11). These isolates have a one base pair difference at position bp 170 (G instead of A) in ITS1 (Fig. 2.12). The ITS sequence of isolates P-M and P-B, collected from *Prunus* spp., was identical to the ITS sequence of the majority of the rose isolates (Fig. 2.11). The ITS sequence of the isolate collected on *Prunus lusitanica* (P-S) was 100% homologous to *Podosphaera tridactyla* (NCBI database, accession nr. AY833655) as published by Cunningham et al. (2005). This powdery mildew showed 51 polymorphic base pairs compared to the ITS1, 5.8S and ITS2 sequences of rose powdery mildew. Isolate P-G, collected on *Prunus avium* showed 12 polymorphisms with the sequence of *P. pannosa* on rose and had 100% homology with the sequence of *P. clandestina* (NCBI database accession nr. AY818344).

In general, in vitro plants showed a higher disease index than the cuttings (Fig. 2.13). While cultivars with a higher resistance in vitro, had a higher in vivo resistance as well.
Table 2.4  Growth of monoconidial powdery mildew isolates on different rose genotypes and *Prunus avium*

<table>
<thead>
<tr>
<th>Powdery mildew isolate</th>
<th>Plant genotype</th>
<th><em>R. hybrida</em> cultivars</th>
<th><em>R. laevigata anemoides</em></th>
<th><em>R. hybrida</em> 'Excelsa'</th>
<th><em>R. wichuraiana</em></th>
<th><em>Prunus avium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>R-A</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R-C1</td>
<td>+</td>
<td>±</td>
<td>±</td>
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<tr>
<td>R-C2</td>
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<td>±</td>
<td>±</td>
<td>-</td>
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<td>R-D</td>
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<td>±</td>
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<td>±</td>
<td>-</td>
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<td>R-F1</td>
<td>+</td>
<td>±</td>
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<td>R-F2</td>
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<td>R-G1</td>
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<td>+</td>
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</table>

z  ‘+’ good development of mycelium and conidia; ‘±’ restricted growth of mycelium and/or conidia; ‘-’ no development of powdery mildew

y  ‘Wettra’, ‘Gomery’, ‘Melrose’ and ‘Ville du Roeulx’

Table 2.5  Development of powdery mildew mycelium and conidia on different rose genotypes

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Mycelium development</th>
<th>Conidia formation</th>
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<tbody>
<tr>
<td>‘Wettra’</td>
<td>+</td>
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<tr>
<td>‘Gomery’</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>‘Melrose’</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>‘Ville du Roeulx’</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>‘Excelsa’</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>R. laevigata anemoides</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>R. wichuraiana</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

z  ‘+’ normal development; ‘-’ poor development
Fig. 2.11 Phylogenetic tree (neighbour-joining) based on the number of base pair differences between powdery mildew isolates

*Podosphaera pannosa* infectious on rose and *Prunus* (group 2)
Fig. 2.12 Nucleotide sequence of rDNA ITS5/ITS4 of *Podosphaera pannosa* isolates growing on rose; the one base pair difference of the isolates virulent on rose and *Prunus avium* is indicated in bold.

![Nucleotide sequence figure]

Fig. 2.13 DI% calculated after scoring of powdery mildew infection on in vitro plants and on cuttings of different cultivars.

![DI% bar chart]

Diversity in rose pathogens
2.3.4 Discussion

In our work, three different groups of powdery mildew could be discriminated among 26 isolates collected from rose and Prunus spp., based on differential plant reactions and ITS sequencing. A first group of 18 isolates was highly virulent on rose and not or very weakly virulent on Prunus avium. A second group of 4 isolates was highly virulent on both rose and Prunus avium. A one base pair difference in the ITS1 sequence (Fig. 2.11 & 2.12) could discriminate these two groups of *P. pannosa* strains. Two isolates of powdery mildew collected on *Prunus laurocerasus* had an ITS sequence identical to the sequence of the isolates only highly virulent on roses (group 1) and could therefore be classified as *P. pannosa*. However, these two isolates of the third group were not able to infect roses.

Previous results (Leus et al., 2002) using eight powdery mildew isolates, could not reveal different pathotypes of powdery mildew collected on roses. These results were based on differential reactions on rose plants. All these isolates were included in this study and are classified in group 1. Linde and Debener (2003) found eight different races in eight isolates tested on ten rose genotypes based on differential reactions. They concluded powdery mildew to have a high racial diversity. Three of these isolates (R-2, R-6 and R-10) were part of this study and clustered together in group 1. A screening for differential reactions depends on the range of host plants used and test conditions. Possibly this explains differences, between pathotypes found by Linde and Debener (2003) and our results.

Takamatsu et al. (2000) mentioned that biotypes of the powdery mildew fungi are strictly restricted to their host plants; changes in host range will directly cause niche separation and thus trigger speciation in powdery mildews. Therefore powdery mildews are often identified by the host plant (Cook et al., 1997; Yarwood, 1978) although many plant species can become infected with more than one powdery mildew species (Boesewinkel, 1980). For example on apricot (*Prunus armeniaca* L.) an infection with *Podosphaera tridactyla* and *Erysiphe prunastri* (syn. *Uncinula prunastri*) could occur simultaneously (Viennot-Bourgin, 1968). Kable et al. (1980) observed that peach trees (*Prunus persica* L.) became infected with powdery mildew when infected rose bushes were in the surroundings; the same observation was made on apricot (Yarwood, 1957). These authors concluded that rose powdery mildew is not strictly host specific. Also *P. leucotricha* from an apple orchard could infect neighbouring peach trees (Ries and Royse, 1978). In all these cases only field observations of suggested cross infections were made. In this work we provide evidence that some rose powdery mildew isolates can infect both rose and *Prunus* in controlled conditions using monoconidial isolates.
Interactions of other diseases occurring both on *Prunus avium* and roses have been described. *Prunus* necrotic ringspot virus isolated from roses showed a close relationship to those collected from *Prunus* when partial coat protein gene sequences were compared. Virus isolates from rose could infect *Prunus*, but not all isolates collected from *Prunus* were infectious to rose (Moury et al., 2001).

The ITS sequence of group 1 isolates was identical to the ITS sequence of rose powdery mildew published by Takamatsu et al. (2000) in the NCBI database. To our knowledge it is the first time the same ITS sequence was also found in powdery mildew isolates (P-B and P-M, group 3) collected from *Prunus* spp. Another sequence of rose powdery mildew published by Cunnington et al. (2003) showed one polymorphism. More polymorphisms were found when our sequence was compared to the data of and Saenz and Taylor (1999). In none of the sequences published the one base pair polymorphism observed in the group 2 isolates (R-D, R-N, R-R and R-P) was described. The isolates identified as *P. tridactyla* and *P. clandestina* collected from *P. lusitanica* and *P. avium*, respectively are commonly found on *Prunus* spp. (Braun, 1987).

In our tests differences in morphological development of powdery mildew on *R. laevigata anemoides* and ‘Excelsa’ were observed. On *R. laevigata anemoides* spread of the fungus was only locally with development of conidia while on ‘Excelsa’ mycelium spread without the formation of conidia (Table 2.5). These observations suggest different mechanisms of partial resistance in roses towards powdery mildew. On *Prunus avium* some of the isolates developed only the first mycelial strands indicating defence mechanisms of the plant only became active after germination and first mycelial development.

Based on present analysis we provided evidence that some *Podosphaera pannosa* isolates are not strictly host specific and can infect both rose and *Prunus* spp. Additionally we have demonstrated that *Podosphaera pannosa* isolates with an identical ITS sequence can differ in their host specificity. Although the rDNA ITS region is an appropriate region to study genetic variation at species level in powdery mildew fungi, and in particular members of the genus *Podosphaera* (Cunnington et al., 2005), it remains to be investigated whether other molecular techniques can distinguish between these *Podosphaera pannosa* types.

Among cultivars and species differences in natural resistance were found. The ranking of the cultivars that was found after artificial infection was in agreement with the general knowledge on resistance of the tested cultivars and species. Results found in vitro were confirmed by the greenhouse test, which proves that an in vitro screening procedure can be used for pathotype screening. Because disease resistance occurs in species, rather than in cultivars, the use of species in rose cultivar breeding is interesting.
BIOASSAYS FOR RESISTANCE SCREENING IN COMMERCIAL ROSE BREEDING

To be able to select disease resistant cultivars, bioassays are needed to screen candidate progenitors, seedlings and candidate varieties. In this chapter screening methods for rose breeding programmes are discussed and information on resistances in progenitors and seedlings are collected. Protocols on detached leaves, seedlings in the greenhouse and in field circumstances were evaluated in the breeding programme itself.

This chapter has been partly redrafted after:


3.1 Introduction

Breeding rose cultivars with fungal resistance is urgent, but unpopular among breeders (de Vries, 2000). Resistance breeding requires adequate assays to screen parent plants and offspring. In commercial garden rose breeding, seedlings are often grown in greenhouses during the first year of selection. Disease resistance is therefore only recorded afterwards when plants are growing outside. Mildew resistance is difficult to evaluate in field circumstances because the extent of infection varies substantially from year to year. Black spot does not occur under normal greenhouse conditions; therefore resistance towards these diseases is usually not evaluated on seedlings. Screening in an early selection stage can enhance selection efficiency. In this chapter procedures to enhance resistance to black spot and powdery mildew in a practical breeding programme are discussed. These methods are applied for resistance screening in parent plants and for the evaluation of seedlings and candidate cultivars. Therefore the cross-breeding method used is described first.

Differences in pathotypes can influence a screening towards resistant plants; the most virulent pathotypes should be used in selection for resistance. In field trials and on large populations in selection it is very difficult to interpret results on reported resistances as other pathotypes can appear in between tests and on different moments and locations. An extrapolation of lab tests to field circumstances is not expedient. Yet if resistance breeding is a prerequisite for new cultivars, a ready to use selection protocol has to be implemented notwithstanding possible disadvantages. With this aim the tests in this chapter were developed. Although disease resistance had always been one of evaluation criteria of candidate varieties in the rose breeding programme at the DvP, it became the most important criterion since 1998. A change in the traditional selection procedure, as described above, was needed. Present aim is the selection of roses with a general good resistance or tolerance for black spot and/or powdery mildew. As a consequence this will lead to plants with horizontal or partial resistance. The search for specific resistance genes resulting in vertical resistances was not intended in the research project.

To be able to select disease resistant progeny, the emphasis was on the development of tests for artificial inoculations of parent plants and offspring. Disease resistance breeding requires the most resistant parent plants and an offspring screening as early as possible. Besides the development of methods to use in practice in a rose breeding programme on large populations, information on resistance in parent plants and seedlings was collected. These results were used to evaluate the testing methods and to start with the application of the methods in the breeding programme itself to create roses with an improved resistance.
3.2 Cross breeding method generally used at the DvP

3.2.1 Hand pollination

At the DvP, parent plants of garden roses used for cross breeding are grown under greenhouse conditions. Crosses are made from April to June to allow the hips to ripen during summer. Every cross is made by hand pollination. On closed flowers (one or two days before opening would occur) all petals are removed except for one. This one petal is left to be able to recognize the prepared flower the next day. To emasculate the flower, and avoid self-pollination, the anthers are excised with a pair of tweezers. The next day, in the morning, the plant is pollinated by direct contact with the pollen of a chosen father plant or by putting the pollen on the stigma with a paint brush. Fresh pollen from fully opened flowers can be used. It is also possible to use pollen stored in a freezer (-20°C).

3.2.2 Seeds

During summer the hips ripen; they are harvested in October. Seeds are removed from the hips and directly sown in trays. A stratification period of 3 to 4 months at 2°C is needed. At the end of January the trays are put in a heated greenhouse, after which the seeds will start to germinate. For seeds of cultivar crossings, germination occurs in the next two months; 35 to 60% of the seeds germinate. For species, the seeds often need a longer germination period or will not germinate unless they had a prolonged stratification period.

3.2.3 First selection on seedlings

In March, when the seedlings have developed two or more leaves, they are planted in the greenhouse. During the next months the seedlings are observed and selection begins. Seedlings can be selected by a positive or a negative selection. When negative selection is applied, all plants with deviating characteristics are pulled out and thrown away. Positive selection means that those plants with the desired characteristics are marked with a coloured stick. At DvP a combination of both methods is used. The first flower of a rose seedling is formed in April-May when the first six leaves are developed. In a classical selection scheme most attention is paid to the flower characteristics of the seedlings. In the first months of selection all aberrant plant and flower types are discarded. In the last months of summer plants with promising features get a coloured stick, indicating they should be used for further selection.
3.2.4 Multiplication and further selection

The selected seedlings are cloned by winter grafting. Of every seedling about 10 grafts are made which are planted outdoors on the field in spring. During the growing season the plants are evaluated for different characteristics. In the classical breeding scheme selection for disease resistance would start at this moment. This selection is highly depended on incidence of diseases in the test field and is therefore influenced by weather conditions. Disease incidence can only be evaluated for pathotypes spontaneously appearing on the field. Further multiplication of desired genotypes is carried out in the field by T-budding in subsequent summers. This selection and multiplication is continued for three or four years. Selected clones are sent to rose contests where they are compared to candidate varieties of other breeders. Only the best genotypes will be commercialized in nurseries. On average 1 of 5000 seedlings will become a commercial cultivar at about 10 years after pollination of the parent plants.

3.3 Black spot resistance screening: natural infections, lab-tests and artificial greenhouse inoculations

3.3.1 Material and methods

3.3.1.1 Natural infection

Black spot has often a slightly different appearance on different rose genotypes. Therefore natural infections of black spot in a rose garden were evaluated on different cultivars and species. In some cases the infection seems limited to little purple spots not developing further towards the characteristic brown-black large leaf spots. Little leaf spots can be confused with other rose pathogens like downy mildew in an early stage or *Elsinoe rosarum*.

In the DvP rose collection, development of black spot was followed from the last week of August 2001 on 4 cultivars: ‘Marie-Louise Velge’, ‘Gomery’, ‘Melglory’ and ‘Slot van Laarne’ and on 6 species and to species related genotypes: ‘Rivierenhof’, ‘Mount Everest’, *R. tomentosa*, *R. canina*, *R. rubiginosa* and *R. × richardii*. During 3 weeks the number of infected leaves was counted on the observed plants, the fourth week this was stopped because too much genotypes dropped their leaves due to black spot infestation. In the genotypes mentioned single leaves were marked and observed weakly during 4 weeks. On every leaf, colour and sizes of the leaf spots were observed. Leaves of ‘Rivierenhof’, ‘Marie-Louise Velge’, ‘Slot van Laarne’, *R. tomentosa* and *R. rubiginosa* were microscopically evaluated on the occurrence of acervuli and conidia. Leaf spots in an early and later stage of development were used.
The occurrence of acervuli was scored:
Score 0 no acervuli
1 very few acervuli
2 an average amount of acervuli
3 many acervuli

To count the amount of conidia, leaf discs (0.7 mm) were made with a cork borer out of the leaf spots. These punches were vortexed in 200 µl of water during 1 min. The amount of conidia in suspension was counted with a haemacytometer (Bürker). Conidia were counted on five different leaf spots for each rose genotype and developmental stage. Mean values were calculated and divided in different classes.

Classes of conidia:
Class 0 no conidia
1 0 to $3 \times 10^3$ conidia/ml
2 $3 \times 10^3$ to $7 \times 10^3$ conidia/ml
3 more than $7 \times 10^3$ conidia/ml

3.3.1.2 Lab-test

A bioassay based on Debener et al. (1998) was used to screen black spot resistance on roses. Two detached leaves were placed in plastic boxes (21 x 11.5 x 5 cm) on moist paper. For every rose genotype 3 boxes were used. Inoculations were made by placing two droplets of 10 µl $2 \times 10^5$ conidia/ml on every leaflet of the leaf. A conidial mixture harvested on infected rose plants was used for inoculation. After 24h, the droplets were removed with a paper tissue. The leaflets were scored individually with a binocular 8 days after inoculation. At least 2 repetitions were made. Minimal 3 leaves with in total at least 18 leaflets were inoculated in the first test, in three other tests 5 leaves with a total of at least 30 leaflets were used. Every leaflet was inoculated on two places. Development of leaf spots and acervuli was evaluated separately by scoring in 3 groups.

Development of leaf spots and acervuli:
Score 0 no infection;
1 leaf spot smaller than the droplet size;
2 leaf spot of size of the droplet.

Development of acervuli:
Score 0 no acervuli;
1 poor development of acervuli;
2 normal development of acervuli.
These scores correspond with respectively resistant, intermediate and susceptible reactions. In total 71 disease free plants were tested for black spot using the detached leaf assay. Because in some cases the reaction of the plant varied a lot in between repetitions and leaves within one repetition, the highest score was taken into account. The quality of black spot conidia was evaluated by use of a phenosafranine solution (100 µl of a 0.1% phenosafranine solution in 300 µl Phosphate buffered Saline (PBS) buffer with 8g/l NaCl, 0,2g KCl, 1,44 g/l Na₂HPO₄, 0,25g/l KH₂PO₄). Pink coloured conidia are considered vital.

3.3.1.3 Greenhouse inoculation

Different rose genotypes were inoculated under greenhouse conditions to evaluate critical inoculation parameters. In normal circumstances black spot does not occur on roses in greenhouse conditions. Black spot needs a prolonged period of 7 h leaf wetness for a good development. It is also known that the fungus spreads within the plant firstly on the older leaves and than upwards with rain splashes (Horst, 1983). The importance of water was evaluated for initial inoculation and further development of the disease. Three cultivars with a known susceptibility for black spot were used: ‘Melgold’, ‘Wettra’ en ‘Ville du Roeulx’; 6 to 9 adult rose plants were used per cultivar. In August all plants were inoculated with 2.10⁴ conidia/ml water of a black spot isolate collected on the field. The conidial suspension was sprayed on all the leaves until ‘run off’.

Treatments:
- Inoculation with and without plastic tent. The tent was used to assure a prolonged period of leaf wetness. The tent was constructed with white plastic foil and the plants were covered during 24 h (Fig. 3.1). The temperature (not measured) in the tent was higher than in the greenhouse. When the tent was removed the leaves were still moist.
- Watering on the leaf or in the pot: to evaluate further development of the disease.

Four groups were formed:
- with tent + watering on the leaves
- with tent + watering in the pots
- without tent + watering on the leaves
- without tent + watering in the pots

The treatment with tent and watering in the pots was not performed on ‘Ville du Roeulx’.
Fig. 3.1 Testplants with and without a tent for black spot inoculation in a greenhouse

The plants were evaluated 14 and 35 days after inoculation. The first date of evaluation gives information on the initial inoculation with and without tent whereas the second date gives more information on the effect of watering on the leaves of the inoculated roses. The plants were scored on a 0 to 3 scale.

Score
0 no leaf spots
1 start of infection on very few leaves
2 infections on about half of the leaves
3 infections on almost all leaves

For every plant a separate score was given to the upper and the lower part of the plant to be able to interpret leaf age and spread of the disease. Out of the results per cultivar, upper and lower part and for every treatment the DI% (see 2.2.3.1) was calculated.

3.3.1.4 Black spot resistance: seedling selection in a greenhouse

To be able to evaluate black spot resistance in an early selection stage in the greenhouse, artificial inoculations were tested. As mentioned before, black spot normally does not occur on greenhouse cultured roses (Horst, 1983). Therefore artificial inoculation was tested at different moments and with different inoculum concentrations on seedlings in a greenhouse.

Moment of inoculation
Five groups of 56 plants were made, each consisting of 4 seedlings from 14 different crosses (Table 3.1 and 3.2). These were planted in a greenhouse in 2004. Three of these groups (groups 1; 2 and 5) were in the same planting bed,
2 were in another (groups 3 and 4). To be able to infect young plants, early in the season black spot was collected the previous year and stored at -20°C. A conidia suspension was prepared by submerging infected leaves in water, the suspension was filtered and the concentration of conidia was counted with a haemacytometer (Bürker). For this test a concentration of 2.10^4 conidia/ml was used. The plants were sprayed with the suspension until 'run-off', for 250 seedlings about 4 l of suspension was used. Under field conditions black spot appears mostly aggressively in the late summer, when the weather conditions are more humid. As the conidia of black spot need water for germination and first development, the seedlings were covered with a tent made of plastic foil during 24 h. Figure 3.2 shows the experimental set-up.

Different groups were sprayed at different dates:
- Group 1: 24/03/04 (stored conidia)
- Group 2: 06/05/04 (stored conidia)
- Group 3: 30/08/04
- Group 4: 30/08/04
- Group 5: no inoculation

After inoculation the plants were scored individually on different dates on a 0 (no black spot) to 3 (leaf drop) scale. Out of the individual scores the DI% was calculated for every group of crosses at the different scoring dates (see 2.2.3.1). Averages for each group per scoring date and for all scoring dates were calculated.

Table 3.1 Parent plants used in crossings to evaluate black spot inoculations on different dates

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Table 3.2  Crossing combinations used in the test

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Concentration of inoculum

In 3 planting beds 7 different progenies with each between 7 to 25 seedlings were planted (Table 3.3 & 3.4). These groups were inoculated with 3 different conidial concentrations: $2 \times 10^2$; $2 \times 10^4$ en $1 \times 10^5$ conidia/ml. The inoculation was performed in the same way as for the tests on the inoculation date. The plants were scored 8, 12 and 30 days after inoculation. Out of the scores the disease index (DI%) was calculated for each group of plants.

Fig. 3.2  Black spot inoculation with a sprayer and construction of a tent with plastic foil on a seedling bed
Table 3.3  Parent plants used in crossings to evaluate conidial concentrations for black spot inoculation

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Table 3.4  Crossing combinations used in the test

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</tbody>
</table>

3.3.1.5  Greenhouse versus field screening

During 2003 and 2004, 153 candidate varieties were evaluated for black spot in a preliminary field screening on respectively 4 (20/8, 3/9, 17/9 and 10/10) and 7 (18/5, 4/6, 17/6, 1/7, 14/7, 28/7 and 18/8) scoring dates. A score was given as described in 3.3.1.3 for groups of at least 4 plants of the same genotype. If the infection was not equal on all plants, the worst score was given.

Selected seedlings of the test on inoculum concentration and tests on the moment of inoculation were multiplied by winter grafting and transplanted on a field in spring 2005. To make a comparison to the greenhouse test, observations were made on the occurrence of black spot on respectively 94 and 36 plants of the crosses M37xA38 and T21xM70 on 01/09/2005. The same scoring system as for the tests in 2003 and 2004 was used. Only in the second year a homogenous spread of black spot can be expected depending on the weather conditions.

3.3.2  Results

3.3.2.1  Natural infection

First symptoms of *Diplocarpon rosae* are seen as little red to brown or purple dots. The characteristic black-brown color appears later. Figure 3.3 shows the
evolution of *Diplocarpon rosae* on leaves of ‘Marie-Louise Velge’, a very susceptible cultivar. There is a clear colour change from red to brown-red. Table 3.5 shows for the 10 genotypes observed the percentage of infected leaves on the plant. In table 3.6 the results for occurrence of acervuli and conidia on 5 rose genotypes are presented. The occurrence of conidia is highly correlated (r=0.98; P<0.01 Spearman’s rho) with the amount of acervuli formed. In small red-purple leaf spots less acervuli and conidia are formed.

In the species and related genotypes small leaf spots with little development of acervuli and conidia lead to a minor spread of the disease in the plant. For some leaf spots it is very difficult to make sure they are caused by black spot as no conidia are formed. Confusion with *Sphaceloma rosarum* is possible as at an early stage the same kind of leaf spots is formed by this pathogen. Leaf spots of *D. rosarum* and *S. rosarum* can appear on the same leaf. In some leaf spots even conidia from both pathogens were observed together.

![Week 1](image1.png)
![Week 2](image2.png)
![Week 3](image3.png)
![Week 4](image4.png)

**Fig. 3.3** Evolution during 4 weeks of the leaf spots caused by *D. rosae* on ‘Marie-Louise Velge’
Table 3.5  Evaluation of the percentage black spot infected leaves on different rose genotypes evaluated on 3 data

<table>
<thead>
<tr>
<th>Rose cultivars</th>
<th>% of infection on the leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 1</td>
</tr>
<tr>
<td>'Marie-Louis Velge'</td>
<td></td>
</tr>
<tr>
<td>Old leaves</td>
<td>20</td>
</tr>
<tr>
<td>Young leaves</td>
<td>30</td>
</tr>
<tr>
<td>‘Gomery’</td>
<td>20</td>
</tr>
<tr>
<td>‘Melglory’</td>
<td>10</td>
</tr>
<tr>
<td>‘Slot van Laarne’</td>
<td></td>
</tr>
<tr>
<td>Old leaves</td>
<td>20</td>
</tr>
<tr>
<td>‘Rivierenhof’</td>
<td>20</td>
</tr>
<tr>
<td>‘Mount Everest’</td>
<td>10</td>
</tr>
<tr>
<td>‘R. tomentosa’</td>
<td>10</td>
</tr>
<tr>
<td>‘R. canina’</td>
<td>20</td>
</tr>
<tr>
<td>‘R. rubiginosa’</td>
<td>10</td>
</tr>
<tr>
<td>‘R. × richardii’</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3.6  Type of leaf spots, occurrence of acervuli and conidia on 5 rose genotypes

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Size and leaf spot colour</th>
<th>Acervuli(^2)</th>
<th>Conidia(^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Rivierenhof’</td>
<td>small - red-purple</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>big - brown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>‘Marie-Louis Velge’</td>
<td>small - red</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>big - dark brown</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>‘Slot van Laarne’</td>
<td>small - purple</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>big - brown</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘R. tomentosa’</td>
<td>small - purple</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>big – dark brown (black)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>‘R. rubiginosa’</td>
<td>small - purple</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>big – dark brown</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^2\) not scorable because of leaf fall

\(^7\) score: 0: no acervuli, 1: very few acervuli, 2: an average amount of acervuli, 3: many acervuli

3.3.2.2 Lab-test

In general, scores of leaf spot (Fig. 3.4) and acervuli development on individual leaflets were highly correlated (r=0.87, p<0.01). However, on some leaves there was a strong development of spots without development of acervuli. In total 7 of the genotypes did not develop acervuli, while 5 plants did not develop leaf spots (Fig. 3.5).
**Fig. 3.4** Infection of black spot on detached leaves 8 days after inoculation

**Fig. 3.5** Distribution of the highest scores for leaf spots and acervuli of black spot on rose seedlings: 0 = resistance, 1 = intermediate reaction, 2 = susceptible (n=71)

**Fig. 3.6** 'Ville du Roeulx' with black spot infection at the first scoring date; the plants were placed under a tent and watered on the leaves after inoculation
3.3.2.3 Greenhouse inoculation

It was possible to infect plants in the greenhouse with black spot (Fig. 3.6). The results based on the DI% are presented in figure 3.7 for every cultivar and for every treatment on the two scoring dates. On the first scoring date plants in the treatment covered with a tent got a high DI% whereas the treatment without a tent hardly showed infection. The second scoring shows the highest DI% for the plants that were placed under a tent and were watered on the leaves. There was no preference observed for younger or older leaves. In the first test more young leaves were infected; at the second scoring date this difference was less clear.

3.3.2.4 Black spot resistance: seedling selection in a greenhouse

Moment of inoculation
The earliest symptoms of black spot on the first inoculated plants (inoculation on 24/03/04) appeared about 1 month after inoculation. The group of seedlings inoculated in March showed symptoms until the end of the growing season but infection reached its maximum in May (Fig. 3.8). Some plants with a mild infection looked free of disease by the end of the season. For the second group of plants inoculated on 06/05/04 the first black spot symptoms were visible 14 days after inoculation. Groups 3 and 4, inoculated at the end of August showed symptoms already one week after spraying of the conidia. In these groups the spread of the disease looked more homogenously and was equal in both groups (Fig. 3.8). A 5th group of plants was not inoculated to observe the spread of black spot in the greenhouse. Although this group was planted on 40 cm distance of the other groups, no symptoms of black spot could be observed.

The average DI% is high for all crosses (Fig 3.9). Also individual scores are high for most genotypes in the different progeny groups (Fig. 3.10 & 3.11). Most variation between infections was observed in the first inoculation test. It was not possible to evaluate differences on different scoring dates for each cross as the number of seedlings was rather low. Most crosses varied in infection between individual seedlings.
Fig. 3.7 Disease indexes (DI%) for 3 different cultivars, 14 (A) and 35 (B) days after inoculation with black spot conidia (2.10⁴ conidia/ml) under different circumstances. ‘Ville du Roeulx’ was not tested in the treatment with a tent and water in the pots.
Fig. 3.8 Evolution of the average DI% for the different groups of seedlings inoculated on different dates; group 1: 24/03/04, group 2: 06/05/04 and group 3 en 4: 1/09/04

Fig. 3.9 The average DI% for black spot on 14 crosses scored after inoculation in a greenhouse
In the crosses 98-2xB32, A37x95-221, B29xB32, C47xA41, K15xM37, M37xH17, M49x95-221 and P55x94-70 seedlings with a score less than 2 occurred (Fig. 3.10). The cross B29xB32 had lower DI% compared to the other crosses for the first and second inoculation (Fig. 3.9). For the inoculations at the end of August this difference disappeared. The inoculations at the end of August showed for all crosses uniformly a very high DI%. These high DI’s% were reached faster than compared to earlier infections scored on different dates (Fig. 3.11).
**Inoculum concentration**

Twelve days after inoculation, the DI% was high for all crosses and all conidial concentrations (Fig. 3.13). Only for the lowest concentration ($2.10^2$ conidia/ml) a significant slower development of black spot was observed when compared to the other two concentrations (t-test; $p<0.05$). No significant difference between the concentrations $2.10^4$ conidia/ml and $1.10^5$ conidia/ml could be observed. The progeny of C48xM70 and the reciprocal cross had the lowest DI% 8 and 12 days after inoculation for the lowest concentration used. The DI% after 8 days is significant lower than after 30 days (t-test; $p<0.01$) (Fig. 3.14).

![Fig. 3.13](image_url)

**Fig. 3.13** Mean DI% for different concentrations (conidia/ml) of black spot conidia, scored 12 days after inoculation (for K15xM50 treated with $2.10^4$ conidia/ml only few seedlings were scorable)

![Fig. 3.14](image_url)

**Fig. 3.14** Mean DI% for 3 concentrations of black spot inoculum at the first and last scoring date
3.3.2.5 Greenhouse versus field screening

After evaluation of black spot in a preliminary field test in 2003 and 2004, no clear correlation between both years was observed ($r=0.22, p<0.01$; Pearson). Probably black spot was not homogenously spread in the field on the first year. In 2004 a severe infection was present on most plants. Climate conditions were more in favour of black spot in 2004 compared to 2003. Therefore scoring was ended earlier in 2004.

In 2005, two crosses were evaluated on the field. Part of the progeny of these crosses was inoculated as a seedling in the greenhouse; within these crosses both groups were evaluated separately on a 0 (no infection) to 3 (leaf drop) scale, the DI% was calculated (Fig. 3.15). For both crosses there is a significant difference (Mann-Whitney, $p<0.01$) in black spot infection on the field between the groups of plants inoculated or not inoculated as a seedling in the greenhouse.

![Fig. 3.15](image)

Fig. 3.15 DI% for genotypes of two crosses evaluated for black spot infection in the field; for each cross a group of genotypes was inoculated as a seedling before propagation in the greenhouse. Treatments with significant differences are indicated with different letters (Mann-Whitney, $p<0.05$)

3.3.3 Discussion

3.3.3.1 Natural infection

The reaction of different rose genotypes after inoculation with *Diplocarpon rosae* has been studied by Knight & Wheeler (1978a) and Blechert & Debener (2005).
These authors describe genotype specific resistances, partial resistance and different resistance mechanisms in roses. These types of reaction are also observed in natural infections where it is known that a lot of rose species possess more resistance compared to rose cultivars. Also in our experiments the number of infected leaves increased faster in cultivars compared to species. This has an effect on the development of acervuli and conidia. In the species and related genotypes small leaf spots with little development of acervuli and conidia lead to a minor spread of the disease in the plant.

Only few genotypes were concluded to have partial resistance to black spot. It is known that there is (almost) no black spot resistance in rose cultivars, but in some species resistance is observed (de Vries, 2000). The use of characterised isolates could reveal more information to specific pathotype resistances as is demonstrated by Debener et al. (1998).

### 3.3.3.2 Lab-test

Most genotypes tested were highly susceptible for black spot. A lot of variation was observed between leaves and repetitions. Different factors play a role in the rose-pathogen interaction like leaf age, quality of the conidia, etc. Enough repetitions are needed to draw conclusions on resistance towards black spot. In natural conditions, black spot spreads upwards from down in plant. However, it was observed that in a detached leaf assay also young leaves can be very susceptible to black spot.

### 3.3.3.3 Greenhouse inoculation

The black spot scores on the first scoring date demonstrated that the use of a tent is indispensable to establish infection under greenhouse conditions. As is discussed by Horst (1983), wet leaves are needed for the conidia to germinate. The second scoring date proved water on the leaves is needed for the spread and development of the disease. Plants that were watered on the leaves showed most progress in development of black spot; even on the plants that were inoculated without the use of a tent, black spot developed relatively well compared with the plants that were not watered on top. On the second scoring date, the DI% did not increase on the young leaves for the cultivars placed under a tent. But, a higher DI% was observed in the lower part of the plants. This is due to re-infection, black spot spread with water on the lower leaves. It was demonstrated that young as well as old leaves could be inoculated. According to Horst (1983) leaves are most susceptible while still expanding. The younger leaves scored high on the first scoring date; these leaves were most easy to inoculate by spraying a conidia suspension.
3.3.3.4 Black spot resistance: greenhouse selection

Moment of inoculation
Tests with the inoculation of seedlings in a greenhouse proved it is possible to establish black spot infection already in spring. Because there is not enough wind or air circulation in the greenhouse there is no spread of the infection to neighbouring plants; also the leaves do not fall of the plant as they would do in outdoor conditions. This results in a lower score under greenhouse conditions compared to outdoor grown plants. More leaf fall was observed with the latest inoculations, this is possible due to the physiological state of the plants at the end of the growing season. With tests earlier in the growing season there is much more variation in plant habitus between individual seedlings; this is not ideal for the scoring system. With the first infection the amount of leaves on the plants varied between 5 and 69. The evolution to a high DI% occurred much faster when inoculations were made later in the season probably because of better (more humid) climatological conditions in the greenhouse. Also in outdoor grown roses the occurrence of black spot is mainly observed at the end of the summer. For all crosses the DI% was high with the used amount of inoculum.

An inoculation at the end of August is preferable because of better growing conditions for the fungus and the availability of enough leaf material. A disadvantage is that the favourable conditions can make the infection pressure for re-infection very high. To be able to recognise plants with partial resistance scoring should be done at about 10 days after inoculation before re-infection can occur.

Inoculum concentration
No clear differences were observed between different crosses in the test. This may indicate that either there was no resistance in the genotypes used, or that the sprayed concentrations are too high to make a good evaluation on partial resistance in the progenies. Only when the lowest concentration of $2 \times 10^2$ conidia/ml was used some resistance was observed in the cross C48xM70 and its reciprocal combination. The concentrations were based on tests for detached leaves in a lab. There is no information available on the infection pressure occurring in natural conditions on roses. Possibly the test can be improved with lower densities of conidia. In an ideal situation a concentration of 100 conidia should be used in different repetitions to avoid some plants escaping from infection. As the infection doesn't spread to neighbouring plants under greenhouse conditions a good inoculation is very important. The moment of scoring (10 days after inoculation) is important since re-infection occurs quickly, even when a lower amount of inoculum is used.
3.3.3.5 Greenhouse versus field screening

Scoring of candidate varieties in a field showed little correlation between 2 succeeding years of evaluation. It was observed that this was mainly caused because black spot infection was not uniformly spread on the field. Also different climatological conditions have an influence, and it can neither be neglected that different pathotypes of black spot may occur.

Perennation of *Diplocarpon rosae* on rose leaves was studied by Knight & Wheeler (1977). Out of our results it seems that the pathogen is passed through by winter grafting, since the progeny of the crosses that were inoculated in the greenhouse showed more symptoms in the field. Roses are mainly propagated by T-budding in summer, it is not clear if also this grafting method can pass black spot infection. For winter grafting a woody stem piece is put on a rootstock, while for T-budding only a leaf axil is used. Also cuttings are used for rose propagation. It seems important to use disease free material to prevent the spread of black spot through freshly propagated plants.

All tests revealed there was no real resistance among all genotypes used; variation in susceptibility only occurred to a very low extend.

3.4 Powdery mildew resistance in parent plants and greenhouse selection versus field evaluation

3.4.1 Material and methods

3.4.1.1 Inoculation tower

To test the resistance in rose genotypes an inoculation tower as described by Linde & Debener (2003) was used. The use of this tower allows infections with characterized powdery mildew isolates. Air pressure is used to disperse the conidia. An inoculation tower was build with an oil barrel without bottom (diam. 55 cm, height 85 cm) (Fig 3.16). On top of the tower a funnel was constructed in the lid. This funnel was connected with a pressure vessel \(10^5\) Pa overpressure), in the connection powdery mildew infected rose leaves could be placed; a net avoided the leaves to be blown in the tower itself. When an air-current is passed, by opening the connection with the pressure vessel, conidia are dispersed in the tower. The tower was placed over 27 Petri-dishes with detached rose leaves. These rose leaves were placed on a medium with 5 g/l microagar and 0.3 g/l benzimidazol to prevent contamination of other fungi on the medium.
The use of the inoculation tower allows a standardised inoculation of detached rose leaves. As the age of the leaves has an influence on susceptibility towards powdery mildew, young just unfolded leaves were used in the tests. To check the conidia density dispersed by the tower, a Petri dish without detached leaves was used as a control. The number of spores was counted on this plate on a defined area with a stereomicroscope (10 to 50x) and the number of spores/cm² was calculated. Inoculated leaves were scored 10 days after inoculation and incubation in a plant growth chamber (22°C; 16L:8D; PPFD=30 µmolm⁻²s⁻¹). Individual leaflets were evaluated and the percentage infected leaf was scored in steps of 10%. An additional class of 5% was added as in some cases it was observed that some colonies only existed of the development of very few mycelial strands out of the conidium according to Linde & Debener (2003).

The scores were translated in classes:

Class 0 no infection;
Class 1 leaflets with a maximum infection of 5 to 10% of the leaf area, often only limited to the development of mycelial strands;
Class 2 leaflets with 20 to 50% infection;
Class 3 leaflets with more than 50% of infected leaf area.

Class 0 represents immunity in leaves; class 1 represents very resistant leaves whereas class 2 grouped leaves with an intermediate reaction, susceptible leaves are class 3.

Fig. 3.16 Inoculation tower for standardised inoculation of detached rose leaves with powdery mildew conidia
To test for the ideal inoculation density five cultivars: ‘Bonica’, ‘Gomery’, ‘Heidetraum’, ‘Jacky’s Favorite’ and ‘Melrose’ were used with three different conidal densities 5; 23 and 101 conidia/cm². A group of 85 plants was tested with a conidial concentration of 20 to 49 conidia/cm² and 179 to 245 conidia/cm². These plants belonged to a private breeding company, therefore these plants are indicated with codes (nr. 1 to 85). For every rose genotype at least three leaves were tested in each repetition. A susceptible rose cultivar (‘Païline’) was included for every inoculation to avoid misinterpretation due to bad conidia quality. The conidia used in the tests were sampled as unidentified greenhouse material.

### 3.4.1.2 Tower screening versus field trial

A comparison between tower infection and field infection was made to validate the use of the tower as a practical instrument for powdery mildew resistance screening. For this purpose, a demonstration project of the “Proefcentrum voor Sierteelt” (PCS) at Destelbergen (Belgium) was used as a field reference. In this project new cultivars commercialised by Belgian rose breeders are evaluated in field observations and are compared to some reference cultivars. Disease resistance is one of the characteristics scored. Twenty cultivars in total are evaluated in 3 repetitions in fields of 3 or 6 plants, according to the plant habitus. The plants were planted in November 2003, first evaluations occurred in 2004 on 3 different dates during the growing season: 10/06/04, 15/07/04 and 15/09/04. Every field was scored on a 0 (no infection) to 3 (very diseased) scale. Young unfolded leaves of these cultivars were harvested for inoculation with the inoculation tower. For every cultivar 4 repetitive inoculation experiments were conducted. On cultivars with various results, 2 more tests were done.

### 3.4.1.3 Greenhouse inoculation

Rose seedlings planted in a control greenhouse were compared with ones grown in a greenhouse in which the seedlings were inoculated artificially to screen resistance in a seedling population in a commercial breeding programme. The seedlings were planted in seedling beds in rows of 4 or 6 plants; the distance in between the rows was 30 cm (Fig. 3.17). The artificial inoculation was performed by planting rootstocks of *R. canina* ‘Pfander’s Canina’ in between the seedlings every 2 m. This rose genotype is very susceptible to powdery mildew (Fig. 3.17) and was inoculated in March by dusting a conidial mixture of powdery mildew harvested on infected greenhouse plants.

Tests were performed in 2002 and 2004. In both years another type of evaluation was used. Progenies from crosses made in 2001 were tested in the greenhouse in 2002. From April on, the seedlings were subjected to a negative selection procedure (Fig. 3.18). Six main criteria were taken into account: occurrence of powdery mildew, malformation of flowers, leafy flower, too few petals, aberrant flower colour and poor plant growth. In the greenhouse with
powdery mildew inoculation and the control greenhouse, respectively 5823 and 1811 seedlings were evaluated.

In 2004 progeny groups of crosses, were divided over both greenhouses. The parent plants used for this test are listed in table 3.7. Groups of 20 or 48 seedlings were planted in each greenhouse. Some plants did not develop well and were not counted. The amount of plants suited for scoring is presented in table 3.8. In 2004 no selection was carried out; all plants were evaluated for powdery mildew on different dates. In the greenhouse with inoculation plants the plants were scored on 14/4, 21/4, 29/4, 12/5, 27/5, 10/6, 25/6 and 28/9. In the greenhouse without inoculation plants, the first symptoms of powdery mildew were observed on 12/5, therefore the plants were only scored on the last 5 scoring dates mentioned.

The plants were scored individually on a 0 to 3 scale adapted from Nicot et al. (2002):

Score 0   no powdery mildew
Score 1   a single colony
Score 2   different colonies on different leaves
Score 3   colonies on most leaves

Scoring was ended when the infection had reached its upper limit. Out of these scores a DI% per offspring group was calculated for every date (see 2.2.3.1). To be able to compare individual seedlings a single value was calculated out of the scores given on different dates. This disease factor (DF%) is calculated by giving a weight according to the scoring date. Plants infected in the beginning of the season are rated with a higher weight because this early infection indicates that the plants are more susceptible than plants infected later in the season under circumstances of a higher infection pressure.

\[
\text{DF}\% = \left( \text{score 12May} \times 3 \right) + \left( \text{score 28May} \times 2 \right) + \left( \text{score 10June} \times 2 \right) + \text{score 25June} + \text{score 28Sept} \times 27 \times 100
\]

(with 27 = the highest sum of scores possible)

Bioassays for resistance screening in commercial rose breeding
Fig. 3.17  Planting of seedlings in a greenhouse (A); infected rootstock ‘Pfander’s Canina’ (B)

Fig. 3.18  Selection in the greenhouse with inoculation plants (A) and without inoculation plants (B)
Table 3.7 Genotypes used in the crossings for the greenhouse and field test

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A36</td>
<td>‘Apricot Nectar’</td>
</tr>
<tr>
<td>A38</td>
<td>‘André Brichet’</td>
</tr>
<tr>
<td>C48</td>
<td>‘Cassandra’</td>
</tr>
<tr>
<td>J26</td>
<td>‘Johann Strauss’</td>
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<td>K15</td>
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<td>‘Trier2000’</td>
</tr>
<tr>
<td>94-70</td>
<td>Unnamed seedling</td>
</tr>
</tbody>
</table>

Table 3.8 Progeny used in two different greenhouses and on the field

<table>
<thead>
<tr>
<th>Crossings</th>
<th>Number of seedlings in the greenhouse with artificial inoculation in 2004</th>
<th>Number of seedlings in the greenhouse without artificial inoculation in 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td>C48xM70</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>M70xC48</td>
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<td>20</td>
</tr>
<tr>
<td>T21xM70</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>K15xM50</td>
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</tr>
<tr>
<td>J26xA36</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>M37x94-70</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>M37xA38</td>
<td>44</td>
<td>48</td>
</tr>
</tbody>
</table>

3.4.1.4 Greenhouse selection versus field evaluation

Preliminary field evaluations were made on 153 seedlings selected in 2002 and planted on the field after vegetative propagation. The occurrence of powdery mildew was observed on 5 scoring dates in 2003 (7/7, 1/8, 20/8, 3/9 and 17/9) and in 2004 (17/6, 1/7, 14/7, 28/7 and 18/8). A DF% was calculated for each genotype. The progenies grown in the greenhouse in 2004 were multiplied vegetatively during winter to 5 plants per seedling and were planted on a field in spring 2005 (Fig. 3.19). Each genotype was scored 3 times during 2005 on 23/6, 28/7 and 28/8. Powdery mildew was scored on a 0 to 3 scale as mentioned for the greenhouse screening, one conclusive score was given for each genotype by two persons. When disease symptoms varied between plants of one genotype, the worst score was used. For each scoring date the DI% (see 2.2.3.1) was calculated for groups of progenies. A disease factor (DF%) was calculated for every genotype over different scoring dates taking into account a weight according to the infection date.

\[
DF\% = \frac{(\text{score 23June} \times 2) + \text{score 28July} + \text{score August 29}}{12} \times 100
\]

(with 12 = the highest sum of scores possible)
3.4.1.5 **ITS sequencing**

ITS sequencing was used to characterise the powdery mildew types in the tests. Four different samples were collected in the greenhouse in 2004. In 2005, 3 isolates were collected on the field. On the rose genotypes evaluated at the PCS in Destelbergen, 3 isolates were collected and analysed. The analyses were performed as described in chapter 2.

### 3.4.2 Results

#### 3.4.2.1 Inoculation tower

The classes for the cultivars tested with different conidial concentrations in the tower are presented in table 3.9 and 3.10. ‘Gomery’ was the most susceptible cultivar in the first test. A higher susceptibility when more inoculum is used was observed for ‘Melrose’, ‘Jacky’s Favorite’ and ‘Gomery’. Only ‘Heidetraum’ stayed free of infection at the different concentrations in this test.

In the test on 85 genotypes with 2 different conidial concentrations, a higher infection pressure led to a higher score on 56% of the plants (if score 3 was not reached with the low inoculum dose). Only in 3 genotypes more inoculum led to an inconsistent lower score (Fig. 3.10). A correlation was calculated between scores with the low and high inoculation density used. A significant correlation (p<0.01) was obtained although, the correlation was not very high (r=0.68, Spearman’s rho). ITS sequencing of the isolates used in the tests proved these isolates belong to group 1 only virulent on roses as explained in chapter 2.

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Fig. 3.19  Rose genotypes planted in the field for evaluation of disease occurrence
Table 3.9  Scores on cultivars inoculated with different conidia concentrations

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Conidia/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>'Bonica'</td>
<td>0</td>
</tr>
<tr>
<td>'Gomery'</td>
<td>2</td>
</tr>
<tr>
<td>'Heidetraum'</td>
<td>0</td>
</tr>
<tr>
<td>'Jacky's Favorite'</td>
<td>1</td>
</tr>
<tr>
<td>'Melrose'</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.10  Infection scores on a collection of 85 seedlings

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Conidia/cm²</th>
<th>Infection class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-49</td>
<td>179-245</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>26</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>27</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

3.4.2.2  Tower screening versus field trial

The results of 6 inoculation experiments are presented in table 3.11; the results of the field evaluation are listed in table 3.12. The cross table 3.13 compares the
results given with both evaluation methods. In the field test 16 plants are given the highest score 3. By use of the tower only 9 of these plants are considered to be very susceptible, the other 6 have a score 2. For the plants with score 2 only 1 is given the same score with the tower, 2 others are given a score 1 and 3. For the 2 plants with a score 1 on the field 1 is even considered to have a score of 3 with the tower.

The comparison of the two testing methods is out of question as different types of powdery mildew were involved. The isolates used in the tower belonged to the group 1 isolates only virulent on roses, as was shown by ITS sequencing (see chapter 2). A characterisation of the powdery mildew collected on the demonstration field revealed two different types. Two isolates belonged to group 1, another was a group 2 isolate (R-P) able to infect rose and *Prunus avium*. This isolate is considered to be very virulent.

Only few cultivars showed a good resistance with both testing methods. ‘Rivierenhof’ scored respectively 0 and 1 with the tower and on the field. ‘Annelies’ showed for both evaluation methods resistance to powdery mildew. All other genotypes were more susceptible.

Table 3.11  Scores of powdery mildew infected leaves of 24 rose genotypes inoculated by use of an inoculation tower

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Infection class</th>
<th>Conidia/cm²</th>
<th>Worst score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>‘Jacky’s Favorite’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Cera’</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Rivierenhof’</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘André Brichet’</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Michelle d’Hoop’</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>‘Toporange’</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>‘Lieve’</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>‘Joke’</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>‘Gold Cup’</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>‘Belbex’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Melrose’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Schneewittchen’</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>‘Annick Boeckx’</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>‘Tenderly’</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>‘Mandarino’</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>‘Citronelle’</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Nicole Verlinden’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Brinessa’</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Bukavu’</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Love Letter’</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>‘Louis’ Double Rush’</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>‘Annelies’</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Dinky’</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>‘Maria Mathilda’</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.12  Powdery mildew scores in a field trial scored on 3 different dates

<table>
<thead>
<tr>
<th>Rose genotype</th>
<th>Infection class scoring date</th>
<th>Worst score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10/06/04</td>
<td>15/07/04</td>
</tr>
<tr>
<td>'Jacky’s Favorite'</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>'Cera'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Rivierenhof'</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>'André Brichet'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Michelle d’Hoop'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Toporange'</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>'Lieve'</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>'Joke'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Gold Cup'</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>'Belbex'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Melrose'</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>'Schneewittchen'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Annick Boeckx'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Tenderly'</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>'Mandarino'</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>'Citronelle'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Nicole Verlinden'</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>'Brinessa'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Bukavu'</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>'Love Letter'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Louis' Double Rush'</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>'Annelies'</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>'Dinky'</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>'Maria Mathilda'</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.13  Cross table for the maximum scores given to 24 rose cultivars evaluated in the field and with the inoculation tower

<table>
<thead>
<tr>
<th>Number of plants in the tower test with score:</th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>0</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plants in the field test with score:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>12</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

3.4.2.3  Greenhouse inoculation

The use of inoculation plants for the selection of resistance in seedlings was evaluated. Figure 3.20 presents the results for selection on 4 dates in 2002. In the greenhouse with artificial inoculation, selection towards resistance became the main discriminating factor, while in the control greenhouse other selection criteria were dominant. In this greenhouse selection pressure for disease resistance was lower and disease development was slower. At the end of the
season in September, 48 plants were retained in the control greenhouse for reasons other than disease resistance, while 71 plants were still disease free and retained for further selection in the inoculated greenhouse.

Fig. 3.20 Selection pressure in a commercial rose breeding programme in 2002. Rose seedling populations were grown in either a greenhouse without (A) or with (B) powdery mildew inoculation. Negative selection was done on different dates.

The results of powdery mildew infection in 2004 on two groups of plants are presented in figure 3.21. Infection on the seedlings in the greenhouse with inoculation plants occurred 1.5 months earlier than in the greenhouse without inoculation plants. The overall mean DI% was respectively maximal 32 and 18%.

At the end of the season, 75% of the seedlings in the control greenhouse were not infected by powdery mildew compared to 31% of the seedlings in the greenhouse with inoculation plants. Different types of resistance were observed. The DI% for each scoring date is shown in figure 3.21; the distribution of the scores for every cross is presented in figure 3.22.

C48xM70 and its reciprocal cross showed a good resistance; for all seedlings in the test, only in a few cases a very low disease incidence was noticed. Some seedlings of the cross T21xM70 were completely disease free. Other seedlings showed scores up to 3 on the third scoring date. Only 32.7% of the progeny of this cross got infected by powdery mildew in the greenhouse with inoculation plants. In the greenhouse without inoculation plants almost no plants of this cross were infected. For K15xM50, only scores ranging from 0 to 2 were noted, no seedlings with score 3 were observed. For the crosses J26xA36, M37x94-70 and M37xA38 the amount of seedlings with different scores were uniformly distributed among all possible scores. Although the scores of the progeny of
M37xA38 were quite low at the first scoring dates, they rose from the third observation date on to the highest level of all crosses. The same was true for M37x94-70; here also the infection increased at the third scoring date. Also in the control greenhouse the infection on these crossings occurred later when compared to the other progenies.

The characterisation of the powdery mildew type in the greenhouse by ITS sequencing proved the inoculum belonged to group 1, only virulent on roses (see chapter 2).

**Fig. 3.21** DI% on different dates for the groups of crossings in the two greenhouses: with and without inoculation plants
Fig. 3.22 Distribution of the scores in function of time for the evaluations of seedlings of different crosses in a greenhouse with and without artificial inoculation. Observations were started 3 weeks after planting of the inoculation plants

3.4.2.4 Greenhouse selection versus field evaluation

A correlation given to 153 genotypes evaluated on the field in 2003 and 2004 was calculated. There was a low correlation between the DF% of both years (r=0.65, p<0.05; Spearman's rho).

On the progenies of the crossings evaluated in the greenhouse in 2004, field infection of powdery mildew was observed in 2005. The DI% for every genotype
evaluated on the field on 3 different dates is presented in figure 3.23. The distribution of the scores is given in figure 3.24. The progeny of the crosses C48xM70 and M70xC48 is most resistant on the field. Of the progeny of T21xM70, 21.5% of the genotypes were disease free on the field. All seedlings of K15xM50 were diseased on the field on the second scoring date, whereas no genotype of this cross was infected on the first scoring date. On the third scoring date the scores were lower due to regrowth without new infection. J26xA36 reached the highest DI% of all crosses tested. Also for the two crosses with M37 as a parent the DI% was high. In the cross M37x94-70 all genotypes were moderately infected until the end of July, whereas with M37xA38 all scores from 0 to 3 were distributed among the progeny already at the end of July.

Genotypes which showed no infection in both greenhouses in 2004, had an average DF% in the field of 12.96% (±15.51). Seedlings with infection in the greenhouse, however, had an average DF% of 36.98% (±22.48) in the field. The difference between these two groups was significant (t-test, p<0.01). When the same comparison is made for the two greenhouses separately, the average DF% for the seedlings without infection in the control greenhouse was 12.50% (±15.01) on the field. For the seedlings that showed infection in the greenhouse the DF% in the field was 38.53% (±22.37). The difference is significant (t-test, p<0.01). A last comparison is made for the greenhouse with inoculation plants. The group of plants without infection in the greenhouse scored a DF% of 13.98% (±16.59) in the field compared to 36.74% (±23.21) for the other group. Again, the difference between both groups is significant (t-test, p<0.01).

For the seedlings scored in both greenhouses in 2004, correlations of the DF% with the field scores of 2005 were calculated (Table 3.14). Especially on the second scoring date the correlation between field scores and greenhouse evaluation is very high. When the correlations between the DF% of all individual plants in the greenhouse and on the field are compared (Tables 3.15 & 3.16) the correlation is lower (r=0.53; p<0.01). An equal result is obtained for the correlation between the DF% of the seedlings in the greenhouse with inoculation plants (r=0.53; p<0.01). The correlation is little bit lower when results in the greenhouse without inoculation plants (r=0.45; p<0.01) are compared to the field scores. When correlations between the scoring dates on the field are compared these are higher when groups of progenies are compared whereas individual scores of rose genotypes are correlated.

ITS sequencing on 3 isolates collected in the field revealed that 1 was a group 1 isolate only virulent on roses. Two isolates belonged to group 2, virulent on rose and *Prunus avium*.
Fig. 3.23  DI% for the progeny groups on the field scored on different dates

Table 3.14  Correlation coefficients (Pearson) between DF% of progenies of different crossings as seedlings in a greenhouse, with or without inoculation plants, and DI% of field infection

<table>
<thead>
<tr>
<th></th>
<th>Greenhouse with inoculation plants</th>
<th>Greenhouse without inoculation plants</th>
<th>Field evaluation (23 June)</th>
<th>Field evaluation (28 July)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse with</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plants</td>
<td>0.85*</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenhouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without inoculation plants</td>
<td>0.83*</td>
<td>0.81*</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Field evaluation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(23 June)</td>
<td>0.97**</td>
<td>0.91**</td>
<td>0.84*</td>
<td>1</td>
</tr>
<tr>
<td>Field evaluation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(28 July)</td>
<td>0.77*</td>
<td>0.74NS</td>
<td>0.98**</td>
<td>0.81*</td>
</tr>
<tr>
<td>Field evaluation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(29 August)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* correlation is significant at the 0.05 level  ** correlation is significant at the 0.01 level  NS not significant
Fig. 3.24  Distribution of the scores for the field evaluations on different scoring dates for progeny of different crossings

Table 3.15  Correlation coefficients (Spearman’s rho) between DF% of individual genotypes as seedlings in a greenhouse with inoculation plants in 2004, and by scoring of field infection in 2005

<table>
<thead>
<tr>
<th></th>
<th>Greenhouse with inoculation plants</th>
<th>Field evaluation (23 June)</th>
<th>Field evaluation (28 July)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse with inoculation plants</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field evaluation (23 June)</td>
<td>0.47**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Field evaluation (28 July)</td>
<td>0.56**</td>
<td>0.62*</td>
<td>1</td>
</tr>
<tr>
<td>Field evaluation (29 August)</td>
<td>0.46**</td>
<td>0.55**</td>
<td>0.78**</td>
</tr>
</tbody>
</table>

* correlation is significant at the 0.05 level  ** correlation is significant at the 0.01 level
Table 3.16 Correlation coefficients (Spearman’s rho) between DF% of individual genotypes as seedlings in a greenhouse without inoculation plants in 2004, and by scoring of field infection in 2005

<table>
<thead>
<tr>
<th></th>
<th>Greenhouse without inoculation plants</th>
<th>Field evaluation (23 June)</th>
<th>Field evaluation (28 July)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse without inoculation plants</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field evaluation (23 June)</td>
<td>0.50**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Field evaluation (29 July)</td>
<td>0.44**</td>
<td>0.55*</td>
<td>1</td>
</tr>
<tr>
<td>Field evaluation (29 August)</td>
<td>0.47**</td>
<td>0.50**</td>
<td>0.79**</td>
</tr>
</tbody>
</table>

* correlation is significant at the 0.05 level   ** correlation is significant at the 0.01 level

3.4.3 Discussion

3.4.3.1 Inoculation tower

Water should be avoided on powdery mildew conidia because this may have a negative effect on the viability of conidia (Sivaplana, 1993 & 1994; Nicot et al., 2002) for inoculation other methods should be used in standardised inoculation experiments. An inoculation tower (Linde & Debener, 2003) can be used for uniform and standardised dispersal of powdery mildew conidia. Another possibility like a vacuum-operated settling tower was developed by Reifschneider & Boiteux (1988), and used with Podosphaera leucotrica on apple genotypes by Janse et al. (1999).

In all tests with the inoculation tower a high variability of scores of individual leaves within one repetition was noticed. Often leaflets of one leaf showed the same high level of infection whereas other leaves of the same cultivar within the same repetition showed no infection at all. The variability between leaves can be due to different leaf ages or physiological conditions although as much as possible fresh unfolded leaves were chosen. On greenhouse and outdoor grown roses Frinking & Verweij (1989) observed the highest percentages of sporulating leaf areas on leaves at 4 to 10 days after unfolding; these leaves are probably infected at a very young stage. On older leaves sporulation is at a very low level and on leaves from 14 days and older, sporulation was not observed any longer. Possible differences in results can be partly explained by the influence of cultural conditions e.g. inevitable use of insecticides, water balance and environmental effects like weather conditions. Often also the reproducibility in between repetitions was low. Enough repetitions are therefore needed to make proper conclusions on powdery mildew infections established by use of the inoculation tower. According to Linde (personal communication) they experienced the same difficulties in their experiments. Linde & Debener (2003) used the inoculation tower to test variability between different powdery mildew isolates.
In our tests an unidentified inoculum mixture was collected in a greenhouse, variation within this inoculum is possible. Analysis of the ITS sequence of the inoculum at different time points indicated that the inoculum used for the tower inoculations belonged to a powdery mildew type referred to as group 1 (see chapter 2). The use of characterised monoconidial isolates in large amounts is difficult as all this material has to be cultured on in vitro rose plants. Besides the type of powdery mildew, the quality of the conidia can also differ. As much as possible conidia of young developing infection sites were used. While controlling the number of conidia on an agar plate, the shape of the conidia is evaluated; conidia without a smooth surface are considered not viable. The development of a rapid and reliable technique to assess the quality of powdery mildew inoculum is not available (Nicot et al., 2002).

Nicot et al. (2002) describe advantages and disadvantages in the use of an inoculation tower in mass inoculation with dry powdery mildew spores. They state that the use of a tower is not adopted for work with large numbers of plants, because the use is slow and somewhat cumbersome. One common drawback of most of these dry inoculation techniques is that calibration of inoculum cannot be done prior to inoculation. Quality and quantity of inoculum deposited onto a plant organ can only be observed after the inoculation process. Moreover these methods may occasionally result in the deposition of clumps or chains of conidia. For the inoculation with the tower young unfolded leaves are used. Genotypes with a high susceptibility only in very young leaves will be evaluated to be susceptible, although the results in the field might be better.

The tests with different inoculation densities showed that the infection rate was mostly higher when more conidia per cm² were applied. The cultivar ‘Gomery’ was the most susceptible among the genotypes tested. This cultivar is known to be highly susceptible. Out of this test it was decided that 20 conidia/cm² would be enough to establish infection on the leaves in the tower. In some tests a higher density of 200 conidia/cm² was used. Linde & Debener (2003) also used a concentration of 200 conidia/cm².

Out of the test with 85 genotypes and 2 inoculum densities it can be concluded that there is a tendency to a higher infection rate when more conidia are used. The use of different conidial densities can help to discriminate plants with partial resistance. Bushnell (2002) mentions that more inoculum not necessarily results in a higher infection rate. Spore densities of 100-400/mm² are not uncommon in physiological experiments; in these tests up to one appressorium is formed per epidermal cell. Infection rates decrease as spore density increases because more resistance is induced by primary germ tubes. Other publications on Leveillula taurica and Podosphaera xanthii on pepper and melon plants show that the aggressiveness of an isolate may be influenced by the density of the inoculum used. For Podosphaera xanthii on melon it was shown that symptoms were uniformly near the maximum severity index with a spore density between 200
and 900 conidia per cm² for different isolates, whereas substantial differences between isolates were observed at a lower density (Nicot et al., 2002).

### 3.4.3.2 Tower screening versus field trial

Results obtained with the inoculation tower were compared to scores given in a field test on 20 cultivars. In general, infection rates on both the field test and the detached leaves inoculated with the tower were high. Therefore it is difficult to discuss the use of the tower to find (partial) resistant plants. More analysis would be needed to have a correct validation. Hsam & Zeller (2002) describe on wheat that tests for mildew resistance can be efficiently carried out on detached leaves or seedlings. Gene postulation using detached leaves or intact seedlings revealed the same patterns of reaction concerning resistant and susceptible infection types. However, plants with intermediate disease responses revealed a tendency towards high infection types on leaf segments compared to intact seedlings. This is difficult to discuss in our tests as it was shown that different types of powdery mildew were involved. Tests on in vitro plants and detached leaves (results not shown) suggest that the isolate (R-P) that was collected on the test field is very virulent when compared to other isolates.

Young leaves, as used for inoculation with the tower, are most susceptible for powdery mildew. This will lead to high scores in a tower test for genotypes with a prompt rising in resistance with leaf age whereas the field score for the same genotype will be lower as on the whole plant more leaf ages are evaluated. The test demonstrates that for some genotypes the reproducibility between repetitions is low. Often this occurs with genotypes with an intermediate reaction; especially for these genotypes with partial resistance the circumstances play an important role like e.g. leaf age. This is probably the case for ‘Dinky’. In the field, this plant is evaluated to have a score 1 as powdery mildew was observed on the youngest leaves as was also seen with the tower test where only very young leaves were evaluated. As the results of the test are influenced by the differences in isolates used in the tower and occurring on the field, it is not correct to make an overall conclusion and comparison between both the results. Additional tests are necessary with one isolate in a field test and in the inoculation tower. However, this is very difficult to work out in practice. Only ‘Rivierenhof’ and ‘Annelies’ showed resistance on both the demonstration field and with the inoculation tower.

The ITS sequencing showed that two different isolates can occur simultaneously in a test field. Linde & Debener (2003) demonstrated by a screening on a differential plant set, that different pathotypes can occur in a small geographical region.
3.4.3.3 Greenhouse inoculation

Our results proved that with the use of inoculation plants it is possible to introduce a homogenous spread of powdery mildew in a seedling greenhouse. By the use of inoculation plants in the greenhouse, first infection occurred much earlier compared to natural infection. Frinking et al. (1987) discuss the spread of fungal spores in a greenhouse where roses are grown; spores of *Lycopodium* sp. remain suspended for quite a long time in a closed glasshouse. Powdery mildew on cut roses needs 1 week to spread the infection all over a greenhouse (Pieters et al., 1994). The spore-dispersal system in a greenhouse is very complex. Velocities of air movement are lower than in open air. This implies that sedimentation of the fungal spores, besides impaction by air currents, plays a more important role in a greenhouse than under field circumstances. Tests with powdery mildew on barley plants placed in a greenhouse showed that activities like work in the greenhouse and sprinkling of water increases the amount of spores dispersed (Frinking & Scholte, 1983).

As shown inoculation plants can lead to a more efficient selection of resistant plants in an early stage of seedling selection. The earlier in the selection process resistance screening can occur, the more cost effective. The use of inoculation plants led to a higher DI% in the greenhouse compared to a control greenhouse. Several reasons cause this high DI%. Firstly, at the early stage when inoculation is done, the seedlings develop a lot of young leaves and the infection can become high. Later in the season, plants develop less young shoots susceptible for powdery mildew what makes that by natural infection the observed DI% will be lower. The time difference between artificial inoculation and natural infection might also explain that the DI% is still rising at the end of the season in the greenhouse without inoculation plants. Secondly, the inoculation plants provide a high infection pressure all over the greenhouse. In the greenhouse without these inoculation plants it was observed that the inoculation spreads more in areas with susceptible plants in the surroundings.

3.4.3.4 Greenhouse selection versus field evaluation

When the results of the two greenhouses (with and without inoculation plants) are compared separately to the field evaluation, both show the same correlation. This means that even in the greenhouse without inoculation plants the seedlings were, although later, evaluated in a representative way. Blazek (2004) evaluated a pre-selection towards powdery mildew (*Podosphaera leucotricha*) in apple seedlings and compared one year of greenhouse selection with ten years of orchard evaluation. A good correlation was only observed between the greenhouse infection on individual seedlings and the first year of evaluation in the orchard. The article gives no information on the possible occurrence of different pathotypes of powdery mildew during the different years.
Our results show that a higher correlation can be found when progeny groups of crosses are compared in different years of observation, instead of individual seedlings. The same conclusion was made by Blazek (2004). Therefore the inoculation of seedlings is important to evaluate valuable parental combinations. In our tests little attention was paid to the evaluation of parent plants used to make the crossings. A test of resistance in parent plants would be useful to make promising crosses.

Conclusions can be made on the resistance of genotypes tested. The progeny of the crosses C48xM70 and M70xC48 is most resistant, as well in the greenhouse test as on the field. These genotypes are promising for further evaluation. Tests on the resistance in the parent plants could reveal if one of both parents is responsible for the resistance in the offspring. The plants of the cross J26xA36 have the highest DI% in the field compared to the other crosses. In the greenhouse tests this cross was at an early stage very susceptible in both greenhouses and was the most susceptible for the greenhouse without inoculation plants, this suggests this cross is very susceptible to powdery mildew even when the amount of inoculum is low. Partial resistance can be recognised in the progeny of the combination K15xM50. The offspring showed only a moderate infection. No genotype of this cross reached the maximal score in the greenhouse or on the field.

It should be remarked that both group 1 and group 2 powdery mildew isolates occurred on the field as was observed by ITS sequencing. Knowledge on the isolates occurring is very important. However, the types of isolates in these kinds of tests can not be controlled.
Resistance in plants can be induced by chemical compounds and plant growth promoting rhizobacteria. Information on induced resistance in roses has been published. The aim of this study is to examine the effect of the salicylic acid analogue BTH, an extract of the giant knotweed (*Reynoutria sachalinensis*) and different plant growth promoting rhizobacteria on powdery mildew resistance in roses. Induced resistance can be of importance in breeding to recognize interesting genotypes.
4.1 Introduction

Disease is a rare outcome in the spectrum of plant-microbe interactions. Plants have evolved a complex set of defence mechanisms that prevent infection and disease in most cases. The battery of defence reactions includes preformed physical and chemical barriers as well as induced defence mechanisms such as strengthening of the cell walls and the production of defence-related molecules. Disease can occur when a pathogen is able to overcome the plant defences, e.g. by either actively suppressing or competing them.

The ability of a plant to respond to an infection is determined by genetic traits in both the host and the pathogen. Some resistance mechanisms are specific for plant cultivars and certain strains of pathogens. In these cases plant resistance genes recognise pathogen-derived molecules resulting from expression of so-called avirulence genes, which often triggers a signal cascade leading to rapid host cell death (hypersensitive response, HR). Such ‘gene-for-gene’ relationships usually lead to highly efficient, but very specific, plant resistance. In contrast, another set of plant resistance mechanisms provides broad-spectrum disease control. The mechanisms involved include preformed physical barriers (cell walls including lignin, waxes, accumulation of anti-microbial metabolites, etc.) as well as inducible mechanisms. Induction of resistance mechanisms occurs locally at the site of attempted penetration (e.g. HR, phytoalexines, cell wall strengthening, etc.) as well as in distant (systemic) parts of the plant (Yamaguchi, 1998).

Induced systemic resistance (ISR) is described as the mode of action of disease suppression by non-pathogenic rhizosphere bacteria, called PGPR (plant growth promoting rhizobacteria) like *Pseudomonas* spp. Priming of pathogen-induced genes allows the plant to react more effectively to the pathogen, which might explain the broad-spectrum action of rhizobacteria-mediated ISR (Verhagen et al., 2004). Phenotypically ISR is similar to systemic acquired resistance (SAR) that is triggered by necrotising pathogens (Bakker et al., 2003). ISR and SAR are sometimes used as synonyms, but whereas SAR requires accumulation of salicylic acid (SA) in the plant, ISR is dependent on responses to ethylene and jasmonic acid in *Arabidopsis thaliana* (Pieterse et al., 1998). Genes encoding PR-proteins are not involved in ISR (Verhagen et al., 2004). On an *Arabidopsis thaliana*-based model using *Pseudomonas fluorescens* WCS417 genetic dissection of the ISR signalling pathway revealed that jasmonic acid and ethylene play a key role after activation of specific sets of defence related genes faster or to a higher level (Pieterse et al., 2003; Verhagen et al., 2004). When different signal transduction pathways are triggered simultaneously in *Arabidopsis thaliana*, disease suppression is enhanced. This suggests that combining bacterial traits that trigger either the SA-, ethylene- or JA-dependent response can improve biological control (Bakker et al., 2003; Meziane et al., 2005). Recent research showed SA not to be involved directly but through siderophores. In other cases SA is involved directly.
Siderophores are iron chelating low-molecular-weight molecules that are secreted by microorganisms and graminaceous plants in response to iron deficiency (Crowley, 2001) to take up iron from the environment (Bakker et al., 2003). Given the essential requirement for iron by almost all living organisms, these compounds are not only important for iron nutrition but are also speculated to function in the ecology of microorganisms in the plant rhizosphere. Siderophores have been studied for their importance in plant disease suppression by mediating nutritional competition for iron and contribute directly to the rhizosphere competence of root colonizing bacteria. The ability to manipulate rhizosphere populations via inoculation with siderophore-producing bacteria to alter iron availability is possible (Crowley, 2001). Some pseudomonads also cause disease suppression by producing a systemic resistance response that is induced by the host plant after colonization by pseudomonads that produce pyoverdine and other salicylate-based siderophores.

Research examining the effects of salicylic acid in causing induced systemic resistance under iron-limiting conditions suggests that salicylic acid itself is a siderophore, since it chelates iron and is released in response to iron stress. *Pseudomonas aeruginosa* 7NSK2 produces three siderophores when it is iron-limited. These include pyoverdine, the salicylate derivative of pyoverdine called pyochelin, and salicylic acid (Buysens et al., 1996; Crowley, 2001). The mutant strain KMPCH is impaired in production of pyoverdin and pyochelin, but still produces salicylic acid (Buysens et al., 1996). *Pseudomonas aeruginosa* KMPCH567 is mutant of strain KMPCH with a reduced production of SA. *P. fluorescens* WCS374, a wild type bacterium produces relatively large quantities of SA under conditions of iron limitation (Bakker et al., 2003). Also *P. aeruginosa* 7NSK2 produces SA (De Meyer & Höfte, 1997). For both, research showed SA to be involved through the siderophores pyochelin and pyocyanin in the case of *P. aeruginosa* 7NSK2 (Audenaert et al., 2002) and pseudomonine with *P. fluorescens* WCS374 (Mercado-Blanco et al., 2001) although the role in ISR of these pathways has not been demonstrated. Other possibilities currently studied are involvement of antibiotics and the role of lipopolysaccharides on ISR. It appears that in most inducing bacteria, more than one determinant is active; therefore the classical way of studying the mode of action by generating specific mutants is not very informative. ISR mediated by *P. putida* WCS358 on *A. thaliana* works through the fluorescent pseudobactin siderophore, the 0-antigen of the LPS, and flagellin; mutants defective in one or two of these three traits still show ISR (Bakker, 2003).

Environmental factors influence the growth of the PGPR and their activity. It is known that the presence of iron in the soil suppresses the production of SA. The temperature does not influence SA production in *P. fluorescens* WCS417 and *P. aeruginosa* 7NSK2 between 28 and 37°C but has an influence on *P. fluorescens* WCS374; more SA is produced if grown at 33°C as compared to 28°C. *P.
*Pseudomonas fluorescens* WCS374 induces ISR at 33 or 37°C but not at 28 or 31°C (Ran, 2002). A high density of bacteria is needed on the roots to obtain ISR. In the study of *Fusarium* on radish, population densities of $10^5$ cfu per gram of root were required to provide biocontrol by *P. aeruginosa* WCS358 which produces siderophores and by *P. fluorescens* WCS374, which causes systemic resistance (Crowley, 2001; Bakker, 2003).

The definition of SAR is often used for chemical compounds such as INA (2,6-dichloroisonicotinic acid) and BTH (benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methylester, BION®; Syngenta, Basel, Switzerland) that can induce resistance. BTH is a chemical SA analogue and induces resistance through the SA dependent pathway (Auchuo et al., 2002). After BTH-treatment in *Arabidopsis thaliana*, there is an early increase of ACC (the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid), followed by a strong induction of SA and a subsequent induction of JA. Free and conjugated forms of SA are found, these induce disease resistance by activation of the SAR signal transduction pathway (von Rad et al., 2005) through SAR-associated PR-genes (Lawton et al., 1996). These PR-genes encode class III chitinases, basic glucanases and acidic peroxidases. BTH shows a long and sustained induction of PR-genes, after 4 days PR-genes are higher than just after treatment on *A. thaliana*. This is essential for a long-lasting resistance (von Rad et al., 2005). Histochemical tests with powdery mildew on wheat proved the germination rate and appressorium formation were not altered by the BTH application, but the penetration rate dropped by to about 30% compared with control plants. The formation of primary and secondary haustoria dropped even more, less than 10% of secondary haustoria were formed compared to the number formed in control plants. The formation of papillae and hypersensitivity responses were seen in the plant cells (Görlach et al., 1996). A disadvantage of BTH is the occurrence of phytotoxicity in some plants at high concentrations, like e.g. on bean (Birigimana & Höfte, 2002).

Positive results resistance about induction by BTH on a wide variety of crops are published although the induction of resistance is dependent on the used pathogen – plant system (Achuo et al., 2004). Due to the fact that the plant activator protects the crops but does not control existing infections, it is important that it is applied before the onset of the diseases (Yamaguchi, 1998).

In roses resistance was induced by use of INA and BTH. The salicylic acid analogue INA could induce resistance against rose powdery mildew (Hijwegen et al., 1996). Also for black spot, resistance induction in roses with BTH was tested with positive results; several mechanisms involved in resistance like extracellular β-1,3-glucanase and chitinase activities were found (Suo & Leung, 2001a&b). Hijwegen et al. (1996) assumed the induction of resistance is more effective in cultivars with partial resistance compared to the induction of resistance in susceptible plants. A basic level of resistance would be necessary to be able to induce the level of resistance in the plant.
Milsana® is the commercial name given to extracts from leaves of the giant knotweed (*Reynoutria sachalinensis* L.). Among Milsana® induced defence responses are peroxidases, β-1,3-glucanases and phytoalexines (Fofana et al., 2002). The application of Milsana® against powdery mildew on cucumber resulted in induced localised resistance through aggregation and collapse of pathogen cells and accumulation of an electron-dense material around the penetrating structures. It was suggested that phenolics are involved in these defence responses by phytoalexines toxic for the fungus (Wurms et al., 1999). On roses Milsana® was tested by Pasini et al. (1997). A limited greenhouse test showed only a partial control of the powdery mildew.

As different authors proved resistance inducing agents can induce resistance in roses to black spot, *Agrobacterium tumefaciens* and powdery mildew, the aim of this study is to examine the effect of the salicylic acid analogue BTH and different plant growth promoting rhizobacteria on powdery mildew resistance in roses. The induction of resistance is thought to be more present in partial resistant plants (Hijwegen et al., 1996), this can be of importance in breeding to recognise interesting genotypes.

### 4.2 Induced systemic resistance for powdery mildew in roses

#### 4.2.1 Material and methods

As a test plant ‘Excelsa’ was used. This plant is known to have some resistance compared to often extremely susceptible cultivars. Two month old cuttings were used. Every test comprised 15 to 24 plants. Treatments were carried out with several resistance inducing agents: BTH, Milsana®, *Pseudomonas aeruginosa*, *P. putida* and *P. fluorescens* strains (Table 4.1). The PGPR were mixed in potting soil in week one at a concentration of 10⁷ bacteria/g soil (Structural 1 universal
(Snebbout) based on sphagnum and garden peat, pH-H₂O: 5-6.5; EC: 325 μS/cm; NPK 14-16-18). Before potting in pots (P9) the roots were dipped in a suspension of 10⁸ CFU/ml. In week three, 15 ml of 10⁸ CFU/ml bacterial suspension was added to each pot. In week five the plants were inoculated with powdery mildew.

The bacteria were grown in Petri dishes on King B-medium (KB):

- **Difco Proteose peptone nr.3**: 20 g
- **Glycerol**: 10 ml
- **K₂HPO₄**: 1.5 g
- **MgSO₄**: 1.5 g
- **Agar**: 15 g
- **Water**: 1 l
- **pH 7.2**

*P. aeruginosa* KMPCH and KMPCH567 were grown on KB with 50 mg/l kanamycine; *P. putida* WCS358, *P. fluorescens* WCS417 and WCS374 were grown on KB with 100 ppm rifampicine. For *P. aeruginosa* 7NSK2 no selective medium was used. The bacteria were incubated overnight at 37°C for the *P. aeruginosa* strains and 28°C for the *P. putida* and *P. fluorescens* strains. By putting 5 ml water on the plates and with a spatula the bacteria were collected. A 1/10 dilution was made and the optical density (OD) was measured with a fotospectrometer (filter 595 nm). OD-value 1 equals 3.5.10⁹ CFU/ml.

For test 1, the inoculation with powdery mildew was done through infected plants close to the test plants that spread the disease. In test 2, a paint brush was used for inoculation and in test 3, plants were inoculated by dusting spores from an infected plant on the test plants. In test 1 and 2 PGPR’s were compared to each other and to different concentrations of BTH. In test 3 the best working PGPR and the highest concentration of BTH was compared to two concentrations of Milsana®. For the treatment with BTH and Milsana® the leaves were sprayed until run off in week one. BTH was tested at 100, 50 and 10 mg/l, for Milsana® 1 ml/l was used. With Milsana® a second treatment was done in week two. In week three the plants were inoculated (Fig. 4.2).

In all the tests every leaf of the plants was scored on a 0 to 5 scale (see 2.3.2) and the disease index was calculated for every plant (see 2.2.3.1). Out of the results a mean disease index is calculated for every treatment. Test 1 was scored 13 times in 55 days time to follow the evolution of the infection; the first score was given after 13 days. Test 2 and 3 were scored three times. Because of the slow start of fungal growth, test 2 was scored three times between 45 and 55 days after start of the inoculation. Test 3 was scored on three dates between 13 and 27 days after inoculation.
Table 4.1  Bacteria and other agents used for resistance induction in roses against powdery mildew

<table>
<thead>
<tr>
<th>Resistance inducing bacteria/chemical compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>7NSK2 (wild type) De Meyer &amp; Höfte (1997)</td>
</tr>
<tr>
<td></td>
<td>KMPCH (SA+ mutant of 7NSK2) De Meyer &amp; Höfte (1997)</td>
</tr>
<tr>
<td></td>
<td>KMPCH567 (SA- mutant of KMPCH) Audenaert et al. (2002)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>WCS358 (wild type) Van Loon et al. (1998)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>WCS417 (wild type) Van Loon et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>WCS374 (wild type) Van Loon et al. (1998)</td>
</tr>
<tr>
<td>BTH (Bion®)</td>
<td>Benzo(1,2,3)thiadiazol-7-thiocarbonacid-S-methylester</td>
</tr>
<tr>
<td>Milsana®</td>
<td>Extract of <em>Reynoutria sachalinensis</em></td>
</tr>
</tbody>
</table>

To control the growth of the PGPR at the roots, the bacteria were plated after the test on selective media. For each plant about 0.2 g of young root-hairs was cut off and grinded in a mortar in 1 ml physiological water, 9 ml more water is added. Of the solution 100 µl is plated out on KB medium with selective antibiotics depending on the bacterial strain. Dilutions 10⁻¹ and 10⁻² also plated. Incubation occurs at 28°C for *P. fluorescens* and *P. putida* strains and at 37°C for the *P. aeruginosa* strains. The plates were incubated during 2 days. The number of colonies was calculated for 1 g of roots.

Fig. 4.2  Treatments with the resistance inducing bacteria, with BTH and Milsana® on cuttings of ‘Excelsa’ and inoculation with powdery mildew
4.2.2 Results

A higher infection occurred on the plants that were inoculated with a paint brush or by dusting conidia, compared to the plants infected by powdery mildew from neighbouring plants (Fig. 4.4). A significant induction of resistance (Mann-Whitney, p<0.05) could be observed for all agents/bacteria except for *P. fluorescens* WCS374 (Fig. 3.4). For the salicylic acid analogue BTH and for Milsana® a dose response effect could be observed in the tests. The effect of induced resistance was the same for the PGPR for the different methods of inoculation resulting in different infection levels (Fig. 4.4). For BTH, the effect of 10 and 50 mg/l was only significant when a high infection level of powdery mildew was applied.

All bacteria were able to colonize the roots with $5 \times 10^5$ CFU/g roots for the tests presented. This concentration is the maximum level of bacteria that can be found for PGPR’s.

![Graph showing infection percentage over days after inoculation](image)

Fig. 4.3 Average DI% for powdery mildew infection in function of the time on ‘Excelsa’ treated with different resistance inducing PGPR and BTH
Fig. 4.4 Mean DI% for ‘Excelsa’ plants treated with PGPR, BTH and Milsana®. Inoculations were made through neighbouring powdery mildew infected plants, scored 55 days after inoculation (A), scored 55 days after inoculation with a paint brush (B) and by dusting the conidia, scored 27 days after inoculation (C). Treatments with significant differences are indicated with different letters (Mann-Whitney, p<0.05)
4.2.3 Discussion

Our results show that all tested bacterial strains, BTH and Milsana® could induce resistance to rose powdery mildew. Only *P. fluorescens* WCS374 did not induce resistance against powdery mildew in 'Exelsa'. Enhanced resistance on roses to powdery mildew was already shown by use of the salicylic acid analogue INA (2,6-dichloroisonicotinic acid) (Hijwegen et al., 1996); BTH is known to enhance resistance in roses against black spot and crown gall (Suo & Leung, 2001b). PR-proteins accumulated after infection of rose plants with *Diplocarpon rosae*; also a treatment with BTH resulted in an accumulation of PR-proteins (Suo & Leung, 2001a & 2002a&b) together with an accumulation of extracellular β-1,3-glucanase and chitinase (Suo & Leung, 2001a). For BTH on wheat against *Erysiphe graminis* f. sp. *tritici* plants treated at the two node stage were protected against powdery mildew for the whole season. The disease symptoms were reduced with 30% compared to a control field. It was found in this research on wheat that the fungus was stopped at penetration and also the formation of haustoria was dramatically lower on treated plants. (Görlach et al., 1996). These results do not indicate that BTH will induce resistance against other diseases on roses or against powdery mildew on other plants as the induction of resistance is dependent on the used pathogen – plant system (Achuo et al., 2004).

Although Milsana® showed the best overall result, the effect of the treatment decreased rapidly one month after treatment (results not shown). When a concentration of 3 ml/l was used there was almost no infection of powdery mildew seen on the leaves. In our test the product was used twice at a one week interval. In contrast to our results, Panini et al. (1997) could only obtain a reduction of 50% of the powdery mildew infection in a preliminary greenhouse test with Milsana®.

Induced systemic resistance was observed for the bacterial strains WCS358 and WCS417 which are known to induce resistance in a SA-independent pathway (Van Loon et al., 1998), and for 7NSK2 and its mutant KMPCH which are known to induce resistance in a SA-dependent pathway (De Meyer & Höfte, 1997). To some extent the mechanism by which the bacteria induce resistance remains unclear. Interestingly, KMPCH-567, a mutant that no longer produces SA (Audenaert et al., 2002), was still able to induce resistance to powdery mildew in rose. Strain WCS347, which did not induce resistance, differs from WCS358 in its lipopolysaccharides (LPS) patterns. In tomato and bean, it was shown that an LPS defective mutant of *P. putida* could not longer induce resistance against *Botrytis cinerea* (Meziane et al., 2005). About the mechanisms of induced resistance through LPS there is not much known (Newman et al., 2001). Another possible reason for the results with strain WCS374 are the temperature conditions this strain requires according to (Ran, 2002): *P. fluorescens* WCS374 induces resistance at 33°C but not at lower temperatures. The temperature in the greenhouse was around 22°C (heated in winter).
Preliminary results with BTH against powdery mildew on roses and cucumber suggest that it is more difficult to induce resistance in cultivars with high disease susceptibility, what will make ISR a tool to recognise basal resistance for breeding purposes (Hijwe gen & Verhaar, 1995; Hijwe gen et al, 1996). In our tests ‘Excelsa’ was chosen as a cultivar with a certain level of resistance. It is not clear whether more susceptible cultivars would respond in a same way to the treatments.

More tests than described above were aimed with PGPR, these are not described because root colonization was poor, probably because of unfavourable environmental conditions. The temperature and moisture in the ground should be sufficient to build up a bacterial colony. Ideal temperatures for *P. aeruginosa* are around 37°C, for *P. putida* and *P. fluorescens* around 28°C. To obtain a good colonization, temperatures in the soil should be high enough. Drought for some days could result in death of the bacteria. Therefore the use of these bacteria in practice is still far away. Some of the bacterial species can be pathogenic for humans, which is unsupportable for practical use.

A large number of rose cultivars, either for cut flower production or garden roses, are grown on a rootstock. For the use of PGPR this would mean they would have to act systemically through the rootstock to have an effect in the leaves. Several tests were performed to define whether or not ISR could work through rootstocks. In none of the tests performed a positive result was observed (results not shown). This could be due to the fact that ISR does not work through rootstocks or might be due to the chosen test plants and rootstock-graft combinations. *P. fluorescens* WCS417 was used on common Canina-type rootstocks ‘Pfander’s Canina’, ‘Schmidts Ideal’, ‘Laxa’ and ‘Inermis’ in combination with the cultivars ‘Ville du Roeulx’, ‘Wettra’, ‘Gomery’ and ‘Melrose’. All these cultivars are highly susceptibility to powdery mildew. No tests were done on ISR on cuttings of these cultivars or rootstocks to test inducibility by PGPR.
Breeding through interploidy crosses

Disease resistance in roses occurs in species rather than in cultivars. The use of species in breeding is hampered by differences in ploidy level. Species are often diploid, whereas most cultivars are tetraploids. To be able to make interploidy crosses in roses ploidy levels of the DvP rose gene pool were analysed. Ploidy in pollen and fertility in triploids are examined, possibilities of crosses to make tetraploids out of diploids through triploids were studied.

This chapter has been partly redrafted after:

5.1 Introduction

5.1.1 Ploidy in rose breeding

In plant breeding, particularly in breeding of ornamentals, ploidy analysis occurs within the general aim of breeding support. Nowadays ploidy analysis is mostly estimated through flow cytometric analysis as an alternative for laborious and time consuming chromosome counts. Four main purposes can be distinguished in flow cytometry for plant breeding: (i) characterisation of available plant material, which includes screening of possible parent plants of a breeding programme as well as evaluation of biodiversity of different populations; (ii) offspring screening after interspecific or aberrant crosses; (iii) ploidy level determination after haploidization and polyploidization treatments, providing a more objective criterium than morphological evaluation and (iv) particle sorting, that allows separation of plant cells based on morphological or fluorescent characteristics (Eeckhaut et al., submitted).

In the genus *Rosa* chromosome numbers are multiples of seven, ranging from 2n=2x=14 to 2n=8x=56. In the four sub-genera of *Rosa*, the three sub-genra *Hulthemia*, *Platyrrhodon* and *Hesperodos* each contain one species in which 2n=2x. In the fourth subgenus, *Europa*, 120 species are divided in 10 sections. In the sections Banksiae, Bracteatae, Chinensis (Indicae), Laevigatae and Synstylae 2n=2x. In the Gallicanae 2n=4x, in the Caroliniae and Pimpennellifoliae 2n=2x and 4x, in the Caninae 2x=4x, 5x and 6x, and in the Cinnamomeae 2n=2x, 4x, 6x and 8x (Darlington & Wylie, 1955).

Modern rose cultivars have a narrow genetic background. Species that contributed to the genepool are mostly diploid, some are tetraploid (see 1.1.2). Modern roses are grouped into horticultural classes that include Polyanthas (2n=2x), Hybrid Teas (2n=3x, 4x), Floribundas (2n=3x, 4x) and Miniatures (2n=2x, 3x or 4x) (Yokoya et al., 2000b). The majority of important cut and garden roses is tetraploid (de Vries & Dubois, 1996). The narrow background of modern rose cultivars make botanical species with valuable traits like disease resistance, cold tolerance or attractive hip formation, interesting to breeding. Interspecific crosses between diploid species and tetraploid cultivars yield triploids. Triploid plants are often assumed to be sterile and to have limited use in breeding programmes. Roses however are known to have some fertility in triploids (de Vries & Dubois, 1996; Grossi & Jay, 2002). There is little information published on interspecific hybridisation in roses. The use of diploids in crossings is still the most obvious way as most species are diploid.

To overcome interploidy problems, different strategies are described to alter the ploidy level of plants. Like in other plants, in roses spindle-formation inhibitors such as colchicines, trifluralin and oryzalin are used to double the chromosome content of diploids (Roberts et al., 1990; Kermani et al., 2003; Zlesak et al.,
Different rose cultivars were bred at the DvP from colchicine doubled *R. rugosa* (Meneve, 1995). Amphidiploids can be produced from tetraploids by using irradiated pollen (Meynet et al., 1994; Crespel et al., 2002). Both procedures gain low success rates in roses compared to other plant species. A rather neglected approach is the use of interploidy crosses.

Although, the aim of this study on interploidy crosses is breeding for resistance, the introduction of new phenotypes remains an interesting opportunity. The introduction of resistant germplasm in rose breeding through rose species will ask for interploidy crosses as most species have a ploidy level different from most cultivars.

Prior to interploidy crosses, different studies were performed on roses:
- Ploidy levels of the DvP rose gene pool were analysed;
- Pollen of diploids, triploids and tetraploids was examined to determine ploidy levels;
- Fertility of pollen in triploids was compared to fertility of pollen from tetraploid plants;
- Possibilities of triploids in crossings and how traits of the diploid plant are transferred to a tetraploid F2 progeny by a triploid F1 were analysed;
- Additional tests on the possibility of ploidy doubling were performed.

### 5.1.2 Ploidy analysis by flow cytometry

Flow cytometry involves the analysis of fluorescence and light-scattering properties of single particles during their passage within a narrow, precisely defined, liquid stream (Galbraith et al., 1983; Dolezel et al., 1989; Dolezel, 1991). Heller (1973) was the first to use this technique for DNA analysis in plants. Galbraith et al. (1983) were the first to use plant nuclei suspensions to examine DNA content and cell cycle in plants after chopping a small amount of fresh tissue in a suitable isolation buffer. This simple, convenient and rapid technique allowed the widespread use of flow cytometry for DNA content related research in different plant species (De Laat et al., 1987; Galbraith, 1990; Arumuganathan & Earle, 1991; Bennett & Leitch, 1995, Bennett et al., 2000, Dolezel, 1997, Dolezel & Bartos, 2005). Compared to ploidy measurements by conventional chromosome counting, flow cytometry offers a valuable, rapid, simple, accurate and fairly cheap alternative. For this reason, DNA flow cytometry has become a popular method for ploidy screening, detection of mixoploidy and aneuploidy, cell cycle analysis, assessment of the degree of polysomaty, determination of reproductive pathways, and estimation of absolute DNA amount or genome size (Dolezel & Bartos, 2005).

Unlike chromosome counts, where the exact amount of chromosomes is determined, a ploidy analysis by flow cytometry is based on the DNA amount in the nuclei. Therefore all measurements are compared to a reference plant of the...
plant species with a known ploidy level. Leaves are the most used plant tissue although other tissues can be analyzed.

The actual preparation of intact nuclei suspensions is almost universally performed following the method of Galbraith et al. (1983). The nuclei are released into an isolation buffer by chopping of a small amount of fresh plant tissue with a razorblade. The nuclei suspension is filtered (e.g. using a pore size of 100 µm). Afterwards, the staining buffer, containing a fluorochrome, is added. The fluorochrome most used for ploidy analysis in plants on a flow cytometer with an arc-lamp is DAPI (4’, 6-diamidino-2-phenylindol) that preferentially binds on A-T. Other possibilities on laser based equipment are Ethidium bromide (EB) and Propidium iodide (PI), that quantitatively intercalate into double-stranded DNA. It may be necessary to neutralize the effect of cytosolic compounds (chloroplast, mitochondria and other debris + soluble substances like phenolics, DNAase, RNAase etc.) using anti-oxidants, proteinases or polyvinyl pyrrolidone (PVP). Also other compounds may be used to prevent interference with DNA staining (Dolezel & Bartos, 2005). After filtration and adding of the fluorochrome, the nuclear suspensions are passed through the flow cuvette, together with a sheath fluid (mostly de-ionized water). The sheath is moving with a greater velocity and forces the sample into a very narrow (10 µm), laminar flowing stream without mixing both streams (hydrodynamic focusing), which allows the individual analysis of the particles.

At a point in the cuvette emitted light of an arc-lamp and/or laser interacts with the sample stream. Light excited by the individual nuclei passing, can be collected as both forward and sideward scattered light. The excitation light may be split into different wavelength intervals by a series of dichroic mirrors. For ploidy analysis one fluorescence parameter is analyzed and translated by a photomultiplier in a electric signal presented as a channel number on a histogram.

The first sample measured in the flow cytometer should be an external standard (control plant). Flow cytometry is a relative method; the first sample gauges the apparatus. The voltage of the photomultiplier, which transfers the fluorescence into an electrical current, is adjusted. The measurement of the external standard should be repeated every 10 measurements to correct the voltage of the photomultiplier if necessary (De Schepper et al., 2001b).

In rose species and cultivars nuclear DNA amounts have been estimated by use of Feulgen scanning microspectrophotometry or flow cytometry (El-Lakany, 1972; Greilhuber, 1988; Dickson et al., 1992; Moyne et al., 1993; Jacob et al., 1996; Yokoya et al., 2000b). Nuclear DNA amounts in roses and other angiosperms are listed by Bennet & Leitch (1995) and Bennett et al. (2000) and in the plant DNA C-values database of Kew botanical garden. Yokoya et al. (2000b) concluded that the 2C amount of diploid roses varies between subgenera, sections and cultivars. Within each section the DNA amounts for
diploid species were similar; also cultivars could be compared to each other. A comparison of genome sizes of the triploid ‘Frensham’ and a diploid *R. pimpinellifolia* results in equal genome sizes. A pentaploid *R. canina* has a larger genome size compared to the hexaploid *R. moyesii*. In the Pimpinellifoliae, DNA amounts of tetraploids were disproportional larger than those of diploid species. Therefore estimations of ploidy levels based on genome sizes can be problematic. The most reliable way to define ploidy levels is a chromosome count. A description of methods to use for chromosome counts in roses is published by Ma et al. (1996). Chromosome counts were published by Grossi & Jay (2002). Moyne et al. (1993) applied flow cytometry on rose tissue to determine regeneration capacity of in vitro callus cultures.

### 5.2 Ploidy in a rose breeding gene pool

#### 5.2.1 Introduction

Polyploidy can arise naturally due to somatic mutation (a disruption in mitosis) resulting in chromosome doubling in (a) meristematic cell(s) that will subsequently produce a polyploid shoot, or due to the merger of unreduced gametes (egg cells or pollen that have undergone First or Second Division Restitution (FDR/SDR). Goldblatt (1980) suggested that 30-70% of flowering plants are of polyploid origin; more recently, 95% of pteridophytes and up to 80% of angiosperms were estimated to be polyploid (Masterson, 1994). Polyploid speciation appears to take place mainly by interspecific hybridization between related taxa, which results in allopoloids or autoallopoloids depending on the degree of relatedness between the hybridizing genotypes. The same taxon may be formed by multiple unrelated hybridization events, which results in a considerable gain in genetic diversity (Soltis & Soltis, 1999). In roses polyploidy arose when mostly diploid species were used to create the first cultivars and evolved through accidental selection to the tetraploid cultivars known today (de Vries & Dubois, 1996).

Knowledge on ploidy levels of parent plants is indispensable information in breeding to make the right combination between seed and pollen parents. Some information on ploidy levels in roses is listed in Modern Roses XI (Roberts et al., 2003). To evaluate the ploidy level of the DvP rose collection, analysis were performed on 339 genotypes.

#### 5.2.2 Material and methods

The protocol used to prepare rose samples for the flow cytometer is based on Otto (1990). Generally the youngest leaf was used for analysis. Older leaves contain more phenolics and other components that might influence the measurement. Rose leaf discs of 250 mm² together with the same amount of young leaf of diploid perennial ryegrass (*Lolium perenne* ‘Isabel’), that was used
as an internal standard, were chopped with a razor blade in 400 µl of a first solution. This first solution consisted of 0.1 M citric acid and 0.5% Tween-20. The internal standard was added because it was experienced that the peak positions of different rose genotypes were not very stable due to the effect of substances interfering with the staining. Self-tanning and other phenolics might interfere with the binding to DNA of fluorochromes used in flow cytometry. Some authors use PVP to bind the phenolics (Yokoya et al., 2000b).

The samples were passed through a nylon filter, mesh 100 µm. The residue on the filter was washed with 700 µl of a solution containing 0.4 M Na₂HPO₄ and 2 mg/l DAPI. For the analysis a CAII and later a PAS III (Partec, Munster Germany) flow cytometer was used. The machine was equipped with an arc lamp and Flomax software. The results are presented as a peak on a histogram with 512 channels. For every analysis peak positions of the rose and grass peak were examined and the peak ratio was calculated. The ratio for every chromosome set for roses is about 0.145 this means the ratio for a diploid plant equals ±0.29, for a triploid this is ±0.33 and ±0.58 for a tetraploid. Depending on the quality of the peaks (expressed as %CV) there might be some aberration on the ratios.

5.2.3 Results

Out of the histograms obtained (example fig. 5.1) the ratio was calculated for every rose genotype analysed and translated in a ploidy level. The results are presented in table 5.1. For some genotypes information on the ploidy level was found in literature (Jacob et al., 1996; Roberts et al., 2003). In some cases ploidy values different from published values were found (Table 5.1).

In table 5.2 genotypes with a ratio that could not be assigned to a ploidy level are listed. Some of the genotypes turned out to be mixoploid (Table 5.3).

5.2.4 Discussion

As expected most cultivars turned out to be tetraploid; in the species more variation occur, a lot of species are diploid. Sometimes the ratio between the rose peak and the peak of the internal standard differed from expected ploidy values and appeared to be in between two ploidy levels. This can indicate aneuploidy or, what is more likely, is caused by genotypical variation in genome sizes that is not linked with the number of chromosomes as discussed by Yokoya et al. (2000b).

In other cases the ploidy level measured with the flow cytometry differed from expected values found in literature. Also here genotypical variation in genome sizes can be the cause, although mislabelling of the plant material is more likely in some particular cases. In the case of R. pimpennelifolii it was discussed by Yokoya et al. (2000b) that the genome size is not correlated with the amount of chromosomes in the same way as for example in rose cultivars. The
measurements made in the study presented here show the same effect as the genome size of R. pimpenellifoliae spp. is evaluated to be triploid based on the peak ratios obtained after flow cytometrical analysis, whereas this species is known to be diploid. Other species show similar aberrations. Some roses turned out to have two ploidy levels in the leaves, viz. combinations of diploid-tetraploid and triploid-tetraploid were found. It was not checked if these ploidy levels could be assigned to the different cell layers in the leaves as would be the case for histogenic ploidy chimerae or whether these are mixoploid within one cell layer. It is remarkable that four of these genotypes are sports, the origin of the other plants is not always clear. Other sports measured showed only one ploidy level. De Schepper et al. (2001a) found somatic polyploidy in flower margins of some azalea sports. The genotypes R. rugosa 1, 2 and 3 are plants that were submitted to a colchicine treatment in the years 1960.

Fig. 5.1 Example of a histogram of a tetraploid rose cultivar measured with an internal standard
Table 5.1 Ploidy levels in the DvP-rose collection

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Rose genotypes</th>
</tr>
</thead>
</table>
Breeding through interploidy crosses

Pentaploid

- 'Agnes', 'Apfelblüte', 'Felicia', 'Highdownensis', 'Louise Odier' (4x), R. canina Kiese, R. rubiginosa, R. laxa inermis, R. macrophylla x moyesii, R. mollis (4x), R. nutkana (6x), R. orientalis, R. primula (2x), R. suffulta, R. tomentosa, 'Seagull', 'Sealing Wax'

Hexaploid


ploidy levels different from literature are marked between brackets (Jacob et al., 1996; Yokoya et al., 2000b; Roberts et al. 2003).

Table 5.2 Genotypes with ratios deviating from the defined ratios at certain ploidy levels

<table>
<thead>
<tr>
<th>Closest ploidy level</th>
<th>Rose genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>'Old Blush', 'Red Wing', R. x pteragonis (2x)², R. stellata mirifica (2x)</td>
</tr>
<tr>
<td>Triploid</td>
<td>'Bouquet Parfait', 'Devoniensis', R. sempervirens (2x – 3x – 4x), R. setigera (2x)</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>'CARMENETTA' (4x), 'Frühlingsanfang', 'Maria Graebner', 'Prairie Dawn', R. californica (2x-4x), R. californica plena (2x), R. fedtschenkoana (4x), R. gallica (4x), R. hemisphaerica (4x), R. holodonta (4x), 'Rosa Mundi' (4x), R. pendulina pyrenaica (4x), R. setigera tomentosa (2x), R. pimpenellifolia (4x), R. virginiana (4x), R. xanthina (2x), 'Stanwell Perpetual' (4x), 'Violacee'</td>
</tr>
<tr>
<td>Pentaploid</td>
<td>R. corymbifera (5x-6x), R. glauca</td>
</tr>
</tbody>
</table>

² ploidy levels in between brackets known ploidy levels according to Jacob et al. (1996); Yokoya et al., 2000b or Roberts et al. (2003) are mentioned.

Table 5.3 Ploidy chimerae

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ploidy levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Céline'</td>
<td>diploid - tetraploid</td>
</tr>
<tr>
<td>'Christine'</td>
<td>diploid - tetraploid</td>
</tr>
<tr>
<td>'Cinderella'</td>
<td>triploid - tetraploid</td>
</tr>
<tr>
<td>'Dream' sport nr. 106*</td>
<td>triploid - tetraploid</td>
</tr>
<tr>
<td>EDVK1*</td>
<td>diploid - tetraploid</td>
</tr>
<tr>
<td>'Goldfink'</td>
<td>triploid - tetraploid</td>
</tr>
<tr>
<td>Nr. 631*</td>
<td>triploid - tetraploid</td>
</tr>
<tr>
<td>R. rugosa 1, 2 and 3</td>
<td>diploid – tetraploid or diploid – triploid</td>
</tr>
<tr>
<td>'Sonja' sport*</td>
<td>diploid – tetraploid</td>
</tr>
</tbody>
</table>

* rose sports
5.3 Pollen ploidy

5.3.1 Introduction

DNA contents of pollen of *Lilium multiflorum*, *Zea mays* and *Chrysanthemum morifolium* were measured by use of a flow cytometer after chopping of the pollen in a protocol described by Bino et al. (1990). In *Lilium* spp. unreduced pollen could be detected by Van Tuyl et al. (1989). Intact rose pollen ploidy can not be measured by flow cytomterical analysis based on DAPI (4′-6′-diamino-2-phenylindole) staining. The exine layer binds DAPI resulting in a strong fluorescent signal of this exine layer. The morphology of rose pollen exine patterns was examined by Ueda & Tomita (1989).

It is assumed that plant cell sizes increase with increasing ploidy levels (Kondorosi et al., 2000); also pollen ploidy levels often show a positive correlation with pollen sizes in homozygote genotypes (Katsiotis, 1995). In some other plants it is assumed that pollen size correlates with flower size (Parnell, 2003; Sarkissian & Harder, 2001). Jacob & Pierret (2000) published results on pollen sizes in roses; these results show that pollen sizes increase with a higher ploidy level in roses. Here a correlation between ploidy levels and pollen diameter was examined by measuring pollen grains.

5.3.2 Material and methods

Open rose flowers of di-, tri- and tetraploid plants were harvested during the flowering season. The ploidy level of these plants was tested (see 5.4). Pollen was harvested by collecting the anthers and dusting pollen on a microscope slide. In some cases the flowers were put 24h under a light bulb to ensure dehiscence. The pollen grains were not wetted for observation. Wetting of the pollen results in swelling of the pollen. The ellipsoid pollens becomes round through absorption of water. Addition of maltose could not prevent swelling of the pollen. In swollen pollen differences between ploidy levels are small compared to the differences in dry pollen (results not shown).

The smallest and largest diameter of 50 pollen was measured for each flower by use of a calibrated ocular. A magnification of 400X was used on an invert microscope (Leica DM IRB). The smallest unit on the graduated ocular scale equalled 1.25 µm. Out of these two diameters the area of an ellips (π.a.b ; with a and b the two radia of the ellips) was calculated; this resembles most the image seen through the microscope. In some cases the flower diameter of the fully opened flower was measured to check a correlation between pollen size and flower size. In a lot of diploid roses the flowers are rather small whether in tetraploid roses the size is bigger. Therefore some genotypes not following this general rule were chosen to measure pollen sizes.
5.3.3 Results

Fig. 5.2 shows the results of the measurements on pollen of di-, tri- and tetraploid plants. For other flowers also the flower diameter was measured (result not shown); there was no correlation found between pollen size and flower size. Pollen of diploid plants is smaller than the pollen of tetraploids, triploids have a higher variation and range in between pollen of diploids and tetraploids. Two groups can be distinguished in pollen of triploid roses (Fig. 5.3). By use of a flow cytometer equipped with a sorting device, pollen fractions differing in size or other morphological characteristics can be sorted in different fractions (Fig. 5.4).

Fig. 5.2 Pollen sizes (area in µm²) of roses with different ploidy levels (n=50)

Fig. 5.3 Diploid and monoploid pollen of a triploid rose cultivar
Fig. 5.4   Triploid roses form monoploid and diploid pollen. The latter, being significantly bigger, can be sorted out of a mixed sample using light scatter parameters. The waste fraction contains smaller pollen grains, beads (reference particles used for the calibration of the machine) and debris; the sorted fraction contains the tallest pollen

5.3.4 Discussion

The size of pollen in roses is related with the ploidy level. Haploid pollen from a diploid plant is smaller compared to diploid pollen from a tetraploid rose. Triploids form pollen with a much higher variation in sizes. The range of pollen sizes of triploid plants indicates that these plants form haploid and diploid pollen. Jacob & Pierret (2000) observed the same variation in pollens sizes notwithstanding these authors measured diameters in wetted pollen. They suggest that pollen of a pentaploid has the same size as pollen in diploids, which is supported by the known haploidy in pollen of pentaploid roses, although this is not supported by the figures they show. Wet pollen expands due to osmotic effects. Whereas dry pollen has an ellipsoid shape, pollen in water becomes spherical. Due to swelling of the pollen, size differences between diploid and haploid pollens become smaller. Experiments with different concentrations of maltose in water could not prevent swelling of the pollen (results not shown). Size differences in pollen of triploids offer possibilities to sort both samples e.g. by sifting or by flow cytometry based on morphological properties of both groups (forward and sideward scatter). Staining of the pollen leads to a loss of viability and is often not useful because of a strong fluorescence of the exine layer. Based on this principal, both sexes in animal and human sperm are sorted after staining with Hoechst 33342 (Johnson et al., 2005; Catt et al., 1997). Preliminary tests with pollen of triploids on a Partec PAS III (equipped with a piezo-electrical sorting device) indicated that an enrichment of a certain pollen fraction based on morphological properties is possible.
5.4 Fertility in triploid pollen

5.4.1 Introduction

In a lot of plants a triploid block prevents crossing with triploids. Often no pollen is formed or when pollen is seen it is sterile. In this test fertility in triploid roses was tested through germination experiments compared to tetraploid plants.

5.4.2 Material and methods

The ploidy level of the plants used was tested (see 5.4).

For germination following medium was used in Petri dishes:
- 40 mg/l $\text{H}_3\text{BO}_3$
- 152 mg/l $\text{CaCl}_2(\text{H}_2\text{O})$
- 150 g/l sucrose
- pH 5.6
- 7 g/l agar

Pollen was put on the medium by dusting from an open flower. Germination was evaluated 24h after incubation at 22°C in the dark. In every Petri dish 200 to 400 pollen grains were evaluated. The percentage germination on the total amount of pollen was calculated. For every cultivar 2 Petri dishes were counted on at least 3 different dates. The tetraploid cultivar ‘Melglory’ was used on every test date as a reference. The results of 11 triploids were compared to two tetraploid cultivars. Besides a germination test, pollen was stained to test the vitality with acetocarmine (2%) and fluorescein-di-acetate (FDA) (0.2% in aceton). For this staining a liquid germination medium as described above was used to hydrate the pollen in an adapted osmotic medium. FDA is added drop by drop until the appearance of the medium becomes turbid. The pollen is suspended on a slide in the medium; 10 minutes after staining the pollen is evaluated microscopically. Together with the staining experiment pollen of the same flower was used to test germination.

5.4.3 Results

The percentage of germination for each cultivar is presented in table 5.4. The percentage in triploid cultivars is never higher than 4%, in the individual results the percentage germinated pollen varied between 0 and 9.83% for the tetraploids this ranged between 0 and 45.37%. In some tests no pollen germinated, probably as a consequence of a bad condition of the flower due to high temperatures. Worst germination was found on ‘View’, pollen of this genotype clustered together; probably the development of this pollen is problematic. For the cultivar ‘Weisse Immensee’ the size difference between
haploid and diploid pollen was still visible on the medium, but not anymore after germination. For every cultivar the acetocarmine stains the highest percentage of pollen. Both stains show a percentage of pollen that is higher than the pollen able to germinate (table 5.5).

**Tabel 5.4** Mean percentages of pollen germinated on artificial medium

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Ploidy (2n)</th>
<th>Mean % germinated pollen</th>
<th>Min – Max %</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Melglory'</td>
<td>4x</td>
<td>22.92</td>
<td>0 – 41.58</td>
</tr>
<tr>
<td>'Maria Mathilde'</td>
<td>4x</td>
<td>43.11</td>
<td>42.20 – 44.04</td>
</tr>
<tr>
<td>'Bingo Meidiland'</td>
<td>3x</td>
<td>3.21</td>
<td>0.74 – 6.93</td>
</tr>
<tr>
<td>'Cera'</td>
<td>3x</td>
<td>2.23</td>
<td>0.74 – 4.47</td>
</tr>
<tr>
<td>'Celina'</td>
<td>3x</td>
<td>1.64</td>
<td>0 – 3.05</td>
</tr>
<tr>
<td>'Heidetraum'</td>
<td>3x</td>
<td>2.77</td>
<td>1.63 – 3.64</td>
</tr>
<tr>
<td>'Jacqueline Humery'</td>
<td>3x</td>
<td>2.19</td>
<td>0 – 3.60</td>
</tr>
<tr>
<td>'Jacky’s Favorite’</td>
<td>3x</td>
<td>1.16</td>
<td>0.41 – 1.70</td>
</tr>
<tr>
<td>'Rivierenhof’</td>
<td>3x</td>
<td>0.36</td>
<td>0.16</td>
</tr>
<tr>
<td>'Rosy Carpet’</td>
<td>3x</td>
<td>1.79</td>
<td>0.74 – 2.35</td>
</tr>
<tr>
<td>'Schnewittchen’</td>
<td>3x</td>
<td>3.71</td>
<td>0.37 – 9.56</td>
</tr>
<tr>
<td>'Schneeflocke’</td>
<td>3x</td>
<td>2.48</td>
<td>2.31 – 3.33</td>
</tr>
<tr>
<td>'View’</td>
<td>3x</td>
<td>0.12</td>
<td>0 – 0.18</td>
</tr>
<tr>
<td>'Weisse Immensee’</td>
<td>3x</td>
<td>0.34</td>
<td>0 – 0.44</td>
</tr>
<tr>
<td>seedling</td>
<td>3x</td>
<td>0.93</td>
<td>0 – 1.52</td>
</tr>
</tbody>
</table>

**Tabel 5.5** Staining and germination (%) of pollen of a diploid, triploid and tetraploid rose

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Ploidy</th>
<th>% acetocarmine stained pollen</th>
<th>% FDA stained pollen</th>
<th>% germinated on medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Mevrouw Nathalie Nijpels’</td>
<td>2x</td>
<td>69.03</td>
<td>56.67</td>
<td>41.67</td>
</tr>
<tr>
<td>'Bingo Meidiland’</td>
<td>3x</td>
<td>33.86</td>
<td>/²</td>
<td>6.93</td>
</tr>
<tr>
<td>'Melglory’</td>
<td>4x</td>
<td>34.23</td>
<td>29.35</td>
<td>14.99</td>
</tr>
</tbody>
</table>

² not tested

**5.4.4 Discussion**

Visser et al. (1977b) obtained up to 64% germination in pollen of tetraploid roses; in our test germination was always lower than 50%. Staining with a 0.1% tetrazolium solution provides a good viability estimate (Visser et al., 1977a&b). In the tests presented here a good estimate was obtained with FDA.

Although pollen fertility in triploids is very low compared to tetraploids, the results demonstrate that a certain amount of viable and germinating pollen is present. This means that triploids are not completely sterile and consequently can be used as crossing parent. It was not investigated to what extent both ploidy levels of pollen in triploids have the same germination capacity.
5.5 Interploidy crosses

5.5.1 Introduction

In spite of their undisputable importance, polyploid plant species are considerable less well studied than their diploid counterparts. This is especially evident for single locus tools like microsatellite DNA analysis. In a diploid plant a maximum of two alleles per locus is expected, the occurrence of one allele is interpreted as the plant being homozygous for that allele. In polyploids the use of microsatellites is not so straightforward (Esselink et al., 2004). In polyploid species, understanding of just how chromosomes are transmitted from one generation to the next is an essential prerequisite for plant genetic studies. Esselink et al. (2004) developed a method to assign precise allelic configurations in tetraploid rose cultivars by use of quantitative values for microsatellite allele peak areas on samples of unknown pedigrees. In dogroses (Rosa sect. Caninae), Nybom et al. (2004) reported up to five alleles in a set of interspecific hybrids.

In rose breeding, especially in the past, breeders had no knowledge on the ploidy levels of used parent plants. As a consequence interploidy crosses are often made and lead to new rose cultivars. In this paragraph a description is made of results of interploidy crosses in a breeding programme, compared to crosses at tetraploid level. Secondly it was investigated whether the use of diploids in crosses with tetraploids can lead to the introgression of genetic material of the diploid into tetraploid cultivars.

5.5.2 Material and methods

5.5.2.1 Crosses

In the breeding programme at the DvP a survey was made on ploidy levels under practical breeding circumstances based on the knowledge of ploidy levels in parent plants as described in 5.2. To that end data were collected on hip and seed formation and on the ploidy level of seedlings of interploidy crosses. Ploidy levels were measured as described in 5.2.2.

5.5.2.2 Plant material

Different interploidy crosses were made by hand pollination. For the genetic AFLP-study the pedigree was used as shown in Fig 5.4. Microsatellites were used on the pedigree shown in Fig. 5.5. For DNA work young leaves were harvested, immediately frozen in liquid nitrogen and stored at -80°C until use.
5.5.2.3 AFLP

DNA was extracted from freeze-dried leaves with a commercial kit (Qiagen DNeasy Plant kit). AFLP fingerprints were generated on an ABI Prism 377 DNA sequencer using Perkin Elmer Biosystems kit for fluorescent fragment detection (Perkin Elmer, 1995). Selective amplification was done with EcoRI and MseI primer combinations (E-AAC – M-CAT). Electropherograms of parent plants, F1 and F2 were compared manually.

5.5.2.4 Microsatellites

Microsatellites were amplified using fluorescently labelled (6-FAM, HEX or NED) primers (Applied Biosystems) from genomic rose DNA according to Esselinck et al. (2003) and Nybom et al. (2005). Allelic configurations of parent plants, F1 and F2 were compared manually.
5.5.3 Results

In interploidy crosses, higher fruit setting and number of seeds per hip occurred in crosses with a tetraploid seed parent, than in crosses with a triploid seed parent (Table 5.6). Flow cytometric analyses of different progenies showed that most seedlings obtained from a cross between a tetraploid and a diploid were triploid. When tetraploids were pollinated with triploids, 98% of the progeny was tetraploid. In crosses between a triploid seed parent and a triploid or tetraploid pollen donor different ploidy numbers were measured (Table 5.6).

Table 5.6 Overview of hip formation, number of seeds per hip and ploidy level of obtained progeny of different interploidy crosses in roses

<table>
<thead>
<tr>
<th>Seed parent</th>
<th>Pollen parent</th>
<th>% hip formation</th>
<th># seeds/hip</th>
<th># tested seedlings</th>
<th>3x</th>
<th>4x</th>
<th>5x</th>
<th>6x</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>4x</td>
<td>56.8</td>
<td>13.8</td>
<td>13</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4x</td>
<td>2x</td>
<td>44.3</td>
<td>5.4</td>
<td>11</td>
<td>8</td>
<td>1</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>3x</td>
<td>4x</td>
<td>23.4</td>
<td>1.1</td>
<td>15</td>
<td>3</td>
<td>11</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4x</td>
<td>3x</td>
<td>43.2</td>
<td>4.7</td>
<td>125</td>
<td>1</td>
<td>123</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3x</td>
<td>3x</td>
<td>14.0</td>
<td>1.1</td>
<td>14</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFLP-analyses of the F1 and F2 generation from an original cross between a tetraploid and a diploid plant showed that that markers are passed on from the diploid species in the F2 plants (Fig. 5.6). The same was observed in the microsatellite analysis, here also some signals are occurring only in the diploid parent plants, the triploid F1 and the tetraploid F2 (Fig. 5.7).

5.5.4 Discussion

Triploid roses used as a pollen parent gave good hip and seed production on the tetraploid mother plant. Fruit set and the average number of seeds per hip are high compared to the germination capability of pollen in triploids. Screening of the obtained progeny showed that almost only tetraploid seedlings are obtained in these crosses. Diploid pollen seems in this case to have a competitive advantage compared to the haploid pollen. Notwithstanding a low efficiency, reciprocal crosses with the triploid plant as a seed parent yields seedlings as well. These results indicate that in roses the so called “triploid block” is not complete. Seeds harvested from the triploid plant, gave both triploid and tetraploid seedlings. This shows that triploid roses form both monoploid and diploid egg cells. Especially if the triploid F1 generation is used as pollen donor efficiency is acceptable. De Vries & Dubois (1996) and Grossi & Jay (2002) also mention the possibility to introduce new characters from diploid species in tetraploid cultivars through triploids resulting again in a tetraploid in two generations of cross breeding only.
Fig. 5.6 Electropherograms obtained after selected restriction fragment amplification by E-AAC and M-CAT of A: ‘Melglory’; B: ‘Guirlande d’Amour’; C: ‘Kasteel van Ooidonk’; D: ‘Jacky's Favorite’; E: 27B; F: 27C. DNA-fragments from the diploid parent are indicated as hatched peaks.

Fig. 5.7 Microsatellite fragments (AB22) obtained of A: ‘Melglory’; B: ‘Guirlande d’Amour’; C: ‘Jacky’s Favorite’; D: ‘Koksijde’; E: 2B; F: 2D and G: 3A. Microsatellite fragments passed by the diploid parent are marked.
When triploids were crossed among each other, triploid and tetraploid seedlings were obtained in equivalent quantities. However, no diploids could be detected in the latter crosses. Probably here fertilisation is also done by diploid pollen, while the egg cells are 50% haploid either 50% diploid. Some of the seedlings had an unexpected ploidy level. Sometimes self-pollination is the cause of unexpected results. In our measurements, a pentaploid was obtained from a cross between a triploid and a tetraploid rose, while hexaploids were found in crosses between a triploid and a tetraploid, and in a cross between two triploid parents. These aberrant ploidy levels may be explained by the formation of unreduced gametes. Production of unreduced gametes in roses is also reported by Crespel and Gudin (2003) and Crespel et al. (2002).

AFLP- and microsatellite data showed that genetic information of a diploid rose can be passed to a tetraploid F2 progeny via a triploid F1. These results demonstrate that the introgression of valuable traits from wild diploid rose species is possible without the use of polyploidization or haploidization protocols. Out of these results it can not be concluded what mechanism plays a role during meiosis. It is possible that recombination occurred by crossing-over among homologue chromosomes or that a whole chromosome is transferred. To analyse the mechanism behind this, PCR reactions on single pollen are needed, or Genomic In Situ Hybridisation (GISH) has to be applied.

As genome sizes between some species and cultivars vary, it is possible that interploidy crosses with these diploid species turn out to be more difficult compared to interploidy crosses with diploid cultivars or species with similar genome sizes. Preliminary experiments on crosses with species showed that results are similar to interploidy crosses with cultivars. More interspecific crosses were described by de Vries & Dubois (2001) between di- and tetraploid species, but also hexa- and octoploid genotypes were crossed with positive results. In breeding with rose species a major disadvantage is loss of favourable characteristics like recurrent flowering in the offspring.
Chapter 6

GENERAL DISCUSSION AND OUTLOOK
In general, plant breeding may be described as: ‘the creation of genotypes for tomorrow with the technology of today by use of yesterday’s gene pool’. In rose breeding the development of disease resistant rose cultivars has become one of the major objectives. In flower crops, breeding for disease resistance started much later than in other sections of horticulture, since most disease problems could be solved chemically. Pressure to reduce or eliminate the use of fungicides and insecticides was less and came later than in food crops (Sparnaaij, 1991). Main pathogens in rose are powdery mildew and black spot. Disease resistance in rose cultivars is very low because in the past, aesthetical properties got priority over other characteristics. In this work the development of auxiliary methodology for fungal disease resistance breeding in roses was aimed at. Pathotypes of powdery mildew and black spot were found and a resistance screening protocol was established for both diseases. Other results in this work were related to the possibilities of induced resistance, and to use interploidy crosses in roses. The establishment of the screening methods led to the first rose genotypes selected for enhanced resistance in the DvP rose breeding programme.

### 6.1 Variation in rose pathogens

Knowledge about the population structure of a pathogen can aid resistance breeding strategies. Pathotype screening of black spot and powdery mildew isolates revealed variation in both pathogens. Results on the occurrence of different pathotypes of pathogens are important for a better understanding of resistance in plant genotypes, as well as to interpret disease resistance screenings.

The genus *Diplocarpon* comprises few species. For the species, *Diplocarpon earlianum*, virulent on strawberry (*Fragaria x ananassa*) different pathotypes were found (Xue et al., 1996). In the study presented here, the variability within the species *Diplocarpon rosae* was studied. Four different black spot pathotypes were found among 6 isolates collected on roses in Belgium. Other authors also described pathotypes of black spot on roses. Five different physiological races could be identified among 15 isolates tested on 10 rose genotypes by Debener et al. (1998). These authors also found one breeding line and the wild species *R. wichuraiana* to be resistant to all isolates tested. Yokoya et al. (2000a) distinguished 4 pathotypes of *D. rosae* on the basis of 16 rose genotypes. One species and one hybrid rose were resistant to all pathotypes tested. In our results it has also been observed that some of the isolates were more virulent than others. The most virulent ones are most interesting for plant resistance screening in a breeding program. *R. wichuraiana* was the only rose with excellent resistance to all isolates in the test as was found by Debener et al. (1998). Tests with different isolates of the virulent black spot isolates are required to evaluate resistance in progenitors and progenies.
For powdery mildew ITS sequences, besides differential plant reactions, were used to differentiate isolates. In recent years, DNA analysis and fingerprinting techniques have increasingly been used to complement pathogenicity characteristics in the analysis of genetic variation in fungi. Due to the obligate biotrophy of powdery mildews, these fungi cannot be cultured on artificial media (Urbanietz & Dunemann, 2005). As a result only a small number of reports about DNA analysis and marker development exist. Most of them are based on barley powdery mildew (Blumeria graminis) (McDermott et al., 1994; Wyand & Brown, 2003). In this study three groups could be discriminated among powdery mildew isolates collected on rose and Prunus spp. A first group showed virulence on rose but not on Prunus avium. A second group, collected on rose genotypes, is virulent on both plant species (4 of 22 powdery mildew isolates collected on rose). This second group can be distinguished from the first group by one base pair difference in the ITS sequence. In the last group, two powdery mildew isolates collected on Prunus laurocerasus would not grow on roses but showed an identical ITS sequence when compared to the isolates only growing on roses. It can be concluded therefore, that a combination of information on the original host plant and the ITS sequence is necessary to group powdery mildew isolates from roses and Prunus. The rDNA ITS region is an appropriate region to study genetic variation at the species level in powdery mildew fungi, and in particular members of the genus Podosphaera (Cunnington et al., 2005). Also for identification of powdery mildew isolates the ITS sequence is used (Cunnington et al., 2003; Cunnington et al., 2004; Kiss et al., 2005). Other sequences to be used for interspecific variation are not yet available. It would be interesting to study whether more genetic variation can be found by sequencing more regions or by use of other techniques like AFLP. In apple, AFLP analysis on a collection of European Podosphaera leucotricha isolates showed an overall low level of genetic variability. Nevertheless a high phenotypic diversity was found when isolates were used to inoculate Malus genotypes (Urbanietz & Dunemann, 2005). Linde & Debener (2003) concluded rose powdery mildew to have a high racial diversity, because all isolates tested by these authors in inoculation tests turned out to be different pathotypes. Probably the set of rose genotypes they used was more differential for different isolates of powdery mildew. For many other powdery mildew species pathotypes are described e.g. for Erysiphe graminis f.sp. hordei on barley, Podosphaera fusca on cucumber (del Pino et al., 2002) and Oidium neolycopersici on Lycopersicon spp. (Lebeda & Mieslerova, 2002).

Differences in powdery mildew development on different rose species were found. On Rosa laevigata anemoides powdery mildew grew only locally with formation of conidia, while on ‘Excelsa’ mycelium growth spread without the formation of conidia. On Prunus avium some of the isolates developed very few mycelia strands. This indicates that the defence mechanisms start after germination and formation of the first hyphae of the fungus. Isolate R-N was the only isolate able to infect Prunus avium and R. laevigata, therefore this isolate is considered the most virulent in our collection. At this moment histochemical
staining is used to study the different resistance reactions in roses towards powdery mildew in more detail. Preliminary results confirm that different resistance reactions in rose genotypes can occur, as was observed on in vitro plantlets in the study presented here. In vitro plants are less suited for disease resistance screening. Scheewe and Ketzel (1994) used in vitro plantlets to test different apple genotypes for resistance to *Podosphaere leucotricha*. Some differences compared to field performance were noticed due to a more receptive leaf surface.

### 6.2 Disease resistance screening

Different methods can be applied for resistance screening for powdery mildew and black spot on individual plants and seedling populations. The earlier in the selection process resistance is screened, the more resistant genotypes will be selected for further evaluation in a cost effective way. Other breeding programs meet the same problems concerning the evaluation of progenies in early selection, at an unreplicated stage in conditions different from normal production (Bradshaw et al., 2003).

Although water can harm powdery mildew conidia (Sivaplana, 1993 & 1994; Nicot et al., 2002) a suspension of powdery mildew conidia was used to spray apple (Battle & Alston, 1996) and roses (Yan, 2005). In the research presented here different methods, avoiding the use of water, were applied on roses. An inoculation tower was evaluated for the screening of parent plants and offspring. This system has also been used for the identification of powdery mildew isolates on apple by Urbanietz & Dunemann (2005) and on rose by Linde & Debener (2003). When this method is used for resistance screening, a large number of repetitions is needed to draw conclusions on the level of resistance in rose genotypes. For large groups of plants this method is too labour intensive. The tower is ideal to test specific genotypes with characterised powdery mildew isolates. Because the powdery mildew fungus is an obligate parasite and cannot be stored for a very long period, mass cultures of monoconidial isolates are difficult to grow. Collections of isolates need to be propagated on in vitro plantlets and have to be sub cultured every month (storage at a lower temperature e.g. 8°C is possible for some months). The problem of pathotype identification in greenhouse and field tests remains problematic. A more detailed identification than the ITS region used in this study, would therefore be appropriate.

Inoculation plants were used to evaluate powdery mildew resistance on seedling populations in a greenhouse. The use of inoculation plants was evaluated positively. It was shown that inoculation plants introduced the pathogen homogenously with a higher infection pressure and earlier in the season when compared to a control greenhouse. Inoculation plants have also been used to infect tomato and tobacco seedlings with *Oidium neolycopersici* (Achuo et al.,
2004; Matsuda et al., 2005) and for crown rust evaluation in perennial ryegrass (Reheul & Ghesquiere, 1996). Seedlings were transplanted from the seed tray to the greenhouse. A different method, leaving the plants at the place of sowing, where they can develop during one season would reduce the amount of work. In the apple breeding program at East-Malling, another method to maintain enough inoculum is applied. There, seedlings were evaluated for powdery mildew by natural infection. If not enough inoculum is present, seedlings are dusted with conidia from mildewed shoots at monthly intervals during the growing season (Alston, 1977). The use of inoculum plants in our methods assured that sufficient inoculum was continuously present. Additional inoculation during the growing season was not necessary.

For both greenhouses, with or without artificial inoculation, correlations for individual genotypes and progenies with field resistance to powdery mildew were calculated. Positive correlations were found between scores of individual genotypes in the greenhouse and on the field. Alston (1983) obtained similar results in apple. In apple breeding yield potential can only be evaluated four years after germination due to the juvenile phase. Therefore, also in apple the inoculation of seedlings was evaluated. For powdery mildew and for scab greenhouse selection is successful and can be evaluated on juvenile seedlings.

Although correlations were found between resistances of individual seedlings in the greenhouse and on the field, these were much higher when the scores of progenies on both locations were compared. Similar results were obtained on apple evaluated for resistance to Podosphaera leucotricha (Blazek, 2004). Therefore it could be interesting to evaluate progeny groups instead of individual seedlings. Also in potato the intense early-generation visual selection between seedlings in a greenhouse and spaced plants at a seed site can be replaced by discarding whole progenies before starting conventional within-progeny selection at the unreplicated small-plot stage. One of the characteristics to be selected for in potato progenies is disease resistance to Phytophthora infestans (Bradshaw et al., 2003). The lower correlation for individual seedlings might be caused by a scoring bias on individual plants, differences in disease development caused by environmental conditions or occurrence of different pathotypes. In the first year of selection only one plant of every genotype is available while in the second selection year about 5 plants are evaluated on the field. The evaluation of progeny groups of one cross can avoid the misinterpretation of resistance on individual seedlings in progenies with a good level of resistance but will not be able to select individual plants in segregating populations.

In strawberry no reciprocal differences were found in breeding for resistance to Podosphaera macularis (Davik & Honne, 2005). In our tests only one reciprocal cross was used. This cross showed a high resistance no matter the crossing direction.
Although black spot does not occur in greenhouses under normal growing conditions, artificial inoculations with black spot on seedlings in a greenhouse environment were possible. The environmental conditions were adapted for successful inoculation experiments; infection was established by using a plastic tent, which resulted in a prolonged period of leaf wetness after inoculation and watering on the leaves. The infection spread most uniformly from the end of August on; earlier in the season conditions seem more unfavourable for the pathogen. The pathogen never infected neighbouring plants in the greenhouse owing to the lack of air flow. On plants inoculated in spring, re-infection led to severe disease symptoms that occurred during the whole season. All genotypes tested showed a very severe disease incidence; it is most likely that resistance in these genotypes was extremely low. To be able to recognise partial resistance in roses a lower inoculum dose should be applied in a repetitive way. At the moment, new progeny groups are evaluated for black spot resistance in a greenhouse by inoculation with a conidial suspension of 100 conidia/ml. Also on strawberry artificial inoculations with a *Diplocarpon* sp. are described. *Diplocarpon earlianum* inoculations on strawberry require the same inoculation conditions compared to *Diplocarpon rosae* on roses. The minimum wetness duration on strawberry is 5 to 15 h. Older leaves develop more symptoms on strawberries (Zheng & Sutton, 1994).

Rose field tests showed a non-homogenous spread of the pathogen could cause problems for correct evaluation in the first year after planting. It is suggested in this study that a greenhouse inoculation can lead to more diseased plants in the field as black spot can probably be transmitted during multiplication by winter grafting. It is not known whether the same occurs with T-budding. Progeny populations in a breeding program of strawberry were evaluated as seedling and adult plant for resistance to leaf scorch (*Diplocarpon earlianum*). The results show a strong correlation between resistance of seedlings and adult-plants. This suggests that strawberry cultivars can be evaluated effectively at the seedling stage (Xue et al., 1997).

To select plants with an enhanced resistance for both diseases, an early inoculation with powdery mildew may be carried out by use of inoculation plants followed by a screening with black spot later in the season. Also for apple selection of powdery mildew five weeks after germination followed by selection for resistance to a second pathogen, scab, six weeks after inoculation is evaluated positively (Alston, 1983). For roses, it is a good idea to cut back the seedlings at the end of July as some plants might slow down vegetative development after formation of the first flower(s). When the plants are cut, more branches will be formed with leaves that can be sprayed to evaluate black spot resistance. After a first year of selection on the seedlings in a greenhouse, the most resistant genotypes can be multiplied and planted outdoors. As for the two main rose diseases: powdery mildew and black spot, pathotypes of the diseases will occur, it will be important to screen the selected genotypes with different
pathotypes. For powdery mildew this can be done by use of an inoculation tower, for black spot detached leaves can be inoculated with a conidial suspension.

Following selection scheme can be advised for a combined selection for resistance screening towards powdery mildew (*Podosphaera pannosa*) and black spot (*Diplocarpon rosae*):

- **Year 0**: resistance of parent plants may be evaluated by use of an inoculation tower for powdery mildew and on detached leaves for black spot;
- **Year 1**: crossings with genotypes promising for disease resistance are made;
- **Year 2**: seedlings are planted in a greenhouse
  - March-July: greenhouse selection for powdery mildew resistance with inoculation plants
  - July: the remaining seedlings are cut back for branching and more leaf development
  - August-September: greenhouse selection for black spot resistance by spraying of a conidial suspension
  - Winter: multiplication of selected seedlings by winter grafting;
- **Year 3**: the multiplied genotypes or groups of progenies are planted outdoors for field evaluation of powdery mildew resistance and other characteristics like flowering;
- **Year 4 and following years**: only from this year on black spot can be evaluated when uniformly spread on the field; genotypes that show an enhanced resistance for powdery mildew and/or black spot in the greenhouse and on the field can be tested with characterised monoconidial isolates on detached leaves. The inoculation tower can be used for powdery mildew and lab infections in plastic boxes can be made for black spot. Tests can be performed until candidate varieties ready for commercialisation or further breeding are selected.

### 6.3 Resistance in roses

In this study a large variation in powdery mildew resistance levels was observed in tested parent plants and seedlings. Although not much specific information was collected on the resistance of the parent plants used in the crossings, some seedlings showed resistance. This creates possibilities with the gene pool used in the breeding programme. It would be interesting to examine the resistance of the parents 'Cassandra', 'Melissa', 'Trier2000' and others used for powdery mildew resistance breeding. This can be done with tests on specific resistance reactions in the plants by use of characterised monoconidial isolates and with more detailed histochemical and microscopical evaluations to evaluate the mechanism of resistance. Work on the interaction of powdery mildew pathotypes on different rose genotypes is in progress. In apple cultivars and species resistance to field infection has been evaluated. In cultivars variation in
resistance, but no immunity is observed (Alston, 1969). The species *M. zumi* and *M. robusta* show immunity to powdery mildew and different major resistance genes were defined (Knight & Alston, 1968). Besides, additional resistance genes are described. Also in strawberry a high variability in powdery mildew resistance among cultivars was found (Davik & Honne, 2005). The experience with powdery mildew in other crops (e.g. barley) implies that continuous breeding is required irrespective of what type of resistance genes have been used. The first resistant strawberry cultivar ‘Korona’ showed an increasing susceptibility to powdery mildew, probably by recombination or mutation in the pathogen resulting in new virulent strains. In other cases resistance appears more durable (Davik & Honne, 2005). Physiological races of powdery mildew on apple were able to overcome the resistance from *M. robusta, M. zumi* and ‘White Angel’. In many other crops similar breakdown of monogenic resistances is reported (Evans & James, 2003).

In some cases the lifetime of a resistant variety is very short. An example is resistance to rust in wheat, where a resistant variety may only be effective for two or three seasons before a new virulent isolate of the pathogens arrives (Ward et al., 1994).

Molecular markers are currently developed for disease resistance screening in roses. Genes for resistance to black spot (*Rdr1*) and powdery mildew (*Rpp1*) have been studied in roses (von Malek et al., 2000; Linde & Debener, 2003; Linde et al., 2004). Marker-trait association has revealed polygenic powdery mildew resistance in tetraploid roses (Yan, 2005). Also in a diploid segregating population qualitative trait loci (QTLs) were mapped for resistance to powdery mildew (Dugo et al., 2005). To target plant disease resistance loci in plants Nucleotidet Binding Site (NBS) profiling can be used (van der Linden et al., 2004). Recently also in *Rosa roxburghii* Resistance Gene Analogues (RGAs) were obtained. These were used to map markers for powdery mildew resistance and can provide information for Marker Assisted Selection (MAS) breeding programs (Xu et al., 2005). Besides the use of markers for selection in crossbreeding, Li et al. (2003) published the development of a transgenic rose line with an enhanced disease resistance. In other Rosaceae marker assisted breeding for resistance to different pathogens is more documented and applied in practice. Within the Rosaceae preliminary results of the comparison between apple and *Prunus* maps suggest a high level of synteny between these two genera. By using the data form different linkage maps anchored with the reference *Prunus* map, it has been possible to establish, in a general map, the position of 28 major genes affecting agronomic characters like disease resistances, found in different species. In apple and *Prunus* markers are currently in use for MAS in breeding (Dirlewanger, 2004).

Within the cross populations tested, no sufficient resistance to black spot was observed. Only in the screening on different pathotypes it was observed that *R. wichuraiana* is resistant to black spot. *R. wichuraiana* is known to have a good resistance towards black spot. For black spot different interaction mechanisms in
rose genotypes are described (Blechert & Debener, 2005). For this pathogen the introduction of new germplasm in the breeding work is indispensable.

Disease resistance to both powdery mildew and black spot is observed in some rose species. Although breeders try to breed with these species, little information on disease resistance breeding with rose species is published. Within the framework of the project ‘Generose’ funded by the European government an evaluation is made on the use of autochthonous European rose species for resistance breeding. Still a lot of rose genotypes which ‘are told’ to have good resistance characteristics should be objectively tested.

Not only powdery mildew or black spot can cause serious damage on roses; rust and downy mildew appear according to environmental conditions. Little research is done on these diseases, while resistance breeding for these pathogens is rarely aimed. For rust different species exist. Determination of these species is very confusing and is often linked to rose species (Shattock, 2003). For downy mildew different pathotypes are observed (Schulz, personal communication). Last years *Elsinoe rosarum* was observed in a number of countries on many rose genotypes (own observations and Linde & Nybom, personal communication). Formerly, this pathogen was rarely observed. Little is known about this disease and no research or resistance breeding is carried out at the moment. A lot of work is still to be done on the pathogens mentioned here, not only for resistance breeding, but also more research on the pathogens themselves is needed.

Resistance in roses towards powdery mildew can be induced with plant growth promoting rhizobacteria, the plant derivative Milsana® or the chemical BTH. On roses, the same effect was obtained for black spot and *Agrobacterium tumefaciens* with BTH by Suo & Leung (2001a & 2001b) and for powdery mildew with another chemical (INA) by Hijwegen et al. (1996). It still needs extra research to investigate whether induced resistance can discriminate plants with different levels of partial resistance in roses. If this is the case, induced resistance could be used to recognise partial resistant plants in breeding. It is suggested that induced resistance can assist powdery mildew resistance breeding in cucumber and rose (Hijwegen & Verhaar, 1995; Hijwegen et al., 1996). Also in barley the plant genotype influences the effect of induced resistance for powdery mildew (Martinelli et al., 1993).

As disease resistance in roses is not always available in the gene pool currently used for breeding, more species should be involved. Most rose cultivars are tetraploid and highly heterozygous. This makes research on specific resistance genes by segregating populations, the use of marker assisted selection, quantitative trait loci and other modern technologies not easy to implement. The introduction of germplasm of resistant (often diploid) rose species into cultivars will be necessary to maintain the position of the rose as a leading ornamental. Interspecific hybridisation has often to cope with ploidy differences. As shown in
this work the use of triploids as an intermediate stage in crosses between diploid species and tetraploid cultivars are useful in the creation of new tetraploid cultivars. In many plant species triploids cannot be used for breeding. In other crops triploids have been used as a bridge between di- and tetraploid genotypes. In Vitis some triploids show female and male fertility (Parker et al., 2002). Several examples exist of interspecific hybridisation among plants with different ploidy levels. A triploid hybrid between Beta vulgaris (4x) and Beta procumbens (2x) showed some fertility and could be used to transfer nematode resistance in the diploid sugar beet. An intergeneric cross between Lolium multiflorum (4x) and Festuca pratensis (2x) resulted in sufficiently fertile triploid hybrids useful in back crosses with diploid L. multiflorum (Thomas, 1993). In Solanum, triploid Solanum commersonii plants were used in crosses with tetraploid potato plants (Solanum tuberosum) and yielded aneuploid pentaploids (Carputo, 2003). Also in lily crosses with triploids, 3x with 2x and 3x with 4x resulted in mostly aneuploid offspring (Lim et al., 2003). The success rates in roses with triploid progenitors are high as seen in pollen fertility of triploids and the progeny obtained in crosses. Molecular markers indicated germplasm of the diploids is passed through the tetraploid level. In some cases only the occurrence of unreduced gametes can explain the ploidy level found in seedlings. Also Crespel & Gudin (2003) made observations on the occurrence of unreduced gametes in roses.

6.4 I promise you a rose garden ...

The ultimate aim of this work involves both research and the development of roses with better fungal resistance. Plants evaluated within the scope of this study, by application of the selection methodology described, are currently evaluated in the field for their potential as candidate cultivars. As the main selection criterion was disease resistance for powdery mildew and/or black spot, the evaluation on aesthetical properties has still to be carried out. The first new cultivars, hopefully combining an enhanced disease resistance with nice flowering characteristics, may be expected within a few years time.
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References


Curriculum vitae

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1991-1995  Industrieel ingenieur Landbouw, Tuinbouw, Industriële Hogeschool van het Gemeenschapsonderwijs CTL, Gent - de grootste onderscheiding
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01/10/99-30/09/04  O&O-project, Min. van Middenstand en Landbouw, DG6, nu IWT-Vlaanderen: 'Ontwikkeling van een biotests voor de screening van rozencultivars en rozenspecies op resistentie tegen echte meeldauw en
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Wetenschappelijke publicaties

Peer reviewed


Andere wetenschappelijke publicaties


**Vulgariserende publicaties**


**Congressen, symposia en studiedagen**

**Deelname met actieve bijdrage**

Applied powdery mildew resistance breeding in roses (voordracht) 18/09/05 – 22/09/05 Fourth international symposium on rose research and cultivation, Santa Barbara - Californië - USA.

Resistance reactions in rose leaves against powdery mildew (*Podosphaera pannosa*) (voordracht) 18/09/05 – 22/09/05 Fourth international symposium on rose research and cultivation, Santa Barbara - Californië - USA.

Identification and occurrence of rust species (*Phragmidium* spp.) on roses in Europe (poster) 18/09/05 – 22/09/05 Fourth international symposium on rose research and cultivation, Santa Barbara - Californië - USA.


Ploidy in plant breeding: from analyzing by flow cytometry to determination by pollen sorting (poster) 23/09/04 – 25/09/04 4th EWGCCA Euroconference 7th EWGCCA Workshop 9th BVC/ABC annual meeting: Flow and Beyond – Cytometry from pathogenesis to Therapy - From Clinical Research to Environmental Biology, Mol.
Evaluation of powdery mildew resistance in a rose breeding program (voordracht) 04/05/04 International Symposium on Crop Protection, Gent.

Schimmelziekten bij roos (voordracht) 13/02/04 Studiedag Boomkwekerij. Min. van de Vlaamse Gemeenschap Administratie Kwaliteit Landbouwproductie Dienst Ontwikkeling, DvP - Melle.

Bioassays for resistance screening in commercial rose breeding (voordracht) 26/08/03 - 29/08/03 Eucarpia 21st International Symposium on Classical versus Molecular Breeding of Ornamentals, Freising - Duitsland.

Molecular evaluation of a collection of rose species and cultivars by AFLP, ITS, rbcL and matK (poster) 26/08/03 - 29/08/03 Eucarpia 21st International Symposium on Classical versus Molecular Breeding of Ornamentals, Freising - Duitsland.

Induced systemic resistance against powdery mildew (*Sphaerotheca pannosa*) on roses (poster) 02/02/03 – 07/02/03 ICPP 8th international Congress of Plant Pathology, Christchurch - Nieuw Zeeland.

Occurrence of pathotypes of *Diplocarpon rosae* on roses in Belgium (poster) 08/09/02 – 14/09/02 EFPP conference ‘Disease resistance in plant pathology’, Praag - Tsjechië.

Interploidy crosses in roses (poster) 03/07/01 – 06/07/01 20th International Symposium Eucarpia – Section Ornamentals, DvP - Melle.

Tools for selection of disease resistant roses (poster) 03/07/01 – 06/07/01 20th International Symposium Eucarpia – Section Ornamentals, DvP - Melle.

Genetic diversity of a collection of rose species and cultivars evaluated by fluorescent AFLP (poster) 27/09/00 – 28/09/00 Forum for applied biotechnology (FAB), Brugge.

Development of a plant bioassay for disease resistance screening in roses (voordracht) 09/05/00 52th International Symposium on Crop Protection, Gent.

Deelname aan internationale vergaderingen, symposia en congressen, zonder actieve bijdrage

2003-2005 Deelname aan verschillende werkvergaderingen voor het Europese project “Generose”: Genetic evaluation of European rose resources for conservation and horticultural use. Key action 5: Quality of Life and Management of Living Resources. Vergaderingen in Melle (België), Ahrensburg (Duitsland), Kristianstad (Zweden) en Marseille (Frankrijk).


25/04/05- 01/05/05 Cost: European training seminar on plant flow cytometry. Geisenheim – Duitsland.

23/11/00 – 26/11/00 Cost 843 meeting ‘Quality enhancement of plant production through tissue culture’ Blankenberge.

09/12/98 – 13/12/98 4th meeting working group 5 Cost 822. Plant regeneration, DvP - Melle.

02/08/98 – 08/08/98 XXV International Horticultural Congress, Brussels.

Begeleiding thesisstudenten


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**Andere activiteiten**

10/02/98 – 15/02/98 Coördinatie van de informatiestand ‘biotechnologisch onderzoek’ die het DvP opzette op Agribex 1998 voor Agrinfo (Secretariaat Generaal Ministerie van Middenstand en Landbouw).