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Comparison of six inoculation techniques with *Colletotrichum acutatum* on cold stored strawberry plants and screening for resistance to this fungus in French strawberry collections

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Abstract

Six inoculation techniques were compared for their ability to evaluate resistance to *Colletotrichum acutatum* of five strawberry cultivars. Inoculation by dipping the whole cold stored plants in a suspension of conidia adjusted to 2.10^6 conidia ml^{-1} made it possible to screen cultivars resistant to crown rot at 28 days after inoculation. Using the dipping technique, 44 strawberry cultivars were evaluated for their resistance to one strain of *C. acutatum*, 1267b. Twelve of them did not show wilt symptoms and could be classified as resistant. When another strain of *C. acutatum*, 494a, was inoculated to seven cultivars, all of them including Dover, resistant to 1267b, showed wilt symptoms. This result showed the importance of investigations on genotype × isolate interactions to conduct an efficient breeding programme for screening resistance to *C. acutatum*.

Introduction

In France, strawberry anthracnose is one of the major diseases affecting strawberry, especially in the south-western area where the humid climate favours the spread of this disease. The predominant species causing anthracnose symptoms on strawberry is *Colletotrichum acutatum* (Denoyes and Baudry, 1995). Symptoms occur on all parts of the plant: petioles, leaves – preferentially young ones – runners, flowers and fruits. Severe losses can be observed in nursery beds and in fields particularly with day-neutral cultivars. So far, no satisfactory disease control method has been developed. The only chemical control treatment reducing losses is dichlofluanide (Euparène, Bayer) which is authorised for Botrytis control as a preventive treatment. The main cultural practice is the use of pathogen-free plants produced according to a scheme of certification (OEPP/EPPO, 1994, Nourisseau, 1986). As these methods do not completely control anthracnose, the use of strawberry cultivar resistance to this disease may be promising to reduce fruit and plant losses. The success of evaluating of germplasm or large populations of strawberry seedlings for resistance to anthracnose depends on the methods used for inoculation and their relationship to field ratings. Several inoculation techniques with *Colletotrichum* spp. have been developed to study the behaviour of strawberry to anthracnose. Spraying is one of the usual methods for screening seedlings, dormant plants or cold stored plants. Inoculum was sprayed over the top of plants until runoff (Simpson et al., 1994, Gupton and Smith, 1991, Smith and Black, 1987, Smith and Spiers, 1982, Delp and Milholand, 1980) or on petioles, making sure however that the inoculum did not run down the petioles into the crown (Delp and Milholand, 1980). Horn and Carver (1963) have inoculated plants by planting them in soil artificially infested with conidia. Other techniques involve wounds, such as injecting inoculum into the crown (Smith et al., 1990) or puncturing the stolon with a needle dipped into an inoculum (Maas and Howard, 1985).

Preliminary inoculation tests using a spraying technique on cold stored plants were not successful in our conditions and did not separate resistant from

susceptible cultivars satisfactorily. Thus, in order to look for discriminant techniques, six techniques were tested on five cultivars using cold stored plants. The most discriminant technique was then used to evaluate 44 strawberry cultivars for resistance to *C. acutatum* (strain 1267b). Moreover, seven of these cultivars were inoculated with 494a, a *C. acutatum* strain, which showed different pathogenicity to 1267b in a previous study.

Materials and methods

Strawberry cultivars

Techniques were evaluated on five strawberry cultivars chosen for their range of resistance/susceptibility to *C. acutatum* (Denoyes and Baudry, 1995): Sequoia and Dover as resistant, Addie as intermediate, Valeta and Elsanta as susceptible.

Susceptibility of 44 cultivars available in French collections were evaluated for their resistance to *C. acutatum* (strain 1267b). At the same time, seven of these cultivars were inoculated with another strain of *C. acutatum*, 494a. Since Osogrande behaved differently according to inoculated strains of *C. acutatum* in a previous experiment (B. Denoyes-Rothan, unpubl.), we chose this cultivar as standard in addition to the susceptible one, Elsanta, and the resistant ones, Sequoia and Dover.

Plants were obtained from three nurseries: Centre Interrégional de Recherche et d'Expérimentation de la Fraise (CIREF), Darbonne and Marionnet. They were purchased as cold stored plants which are available about eight months per year.

Inoculum preparation

Two strains showing different pathogenicity (Denoyes and Baudry, 1995) were inoculated. Strain 1267b did not cause wilting on the resistant cultivars Dover and Sequoia while strain 494a did. Stock culture of strains 1267b and 494a (*C. acutatum)* were maintained on silica gel at 4 °C (Perkins, 1962). Each year, strains were recovered from diseased plants to revive their pathogenicity. Conidial suspensions adjusted to 10^6 or 2.10^6 conidia ml^{-1} according to the inoculation techniques were prepared as previously described (Denoyes and Baudry, 1995).

Inoculations

Six inoculation techniques were tested. (1) In the spot technique, 300μl of an inoculum suspension adjusted to 2.10^6 conidia ml^{-1} was deposited on the rhizome. For 24 h after inoculation, plants were laid flat on a table and protected by wet paper. (2) In the spraying technique, plants were grown in pots for one week before inoculation in order to develop young leaves. The conidial suspension adjusted to 2.10^6 conidia ml^{-1} was applied above the plant until run-off with a hand-pump sprayer held directly at 20 cm. (3) When plants were inoculated by dropping, 150μl of conidial suspension adjusted to 2.10^6 conidia ml^{-1} was put on the scar left by one removed leaf. A hypodermic needle was then introduced through the drop into the crown. (4) Injection inoculation was carried out using a hypodermic syringe to infiltrate crowns with approximately 300μl of the conidial suspension adjusted to 2.10^6 conidia ml^{-1}. (5) and (6) In the last two dipping inoculation techniques, entire plants were immersed in a conidial suspension adjusted to 10^6 or 2.10^6 conidia ml^{-1} respectively for 30 mn. These six techniques were tested in two experiments. Two techniques, spraying and dipping in 10^6 conidia ml^{-1}, were conducted in both experiments while the others were not repeated.

In the study of cultivar susceptiblity, plants were inoculated by dipping in a conidial suspension adjusted to 2.10^6 conidia ml^{-1} for 30 mn. Seven experiments were set up from 1992 to 1994. All cultivars were evaluated at least in two different experiments. The four standard cultivars, Elsanta, Osogrande, Sequoia and Dover, were inoculated in each screening test.

In each experiment concerning techniques or cultivar susceptibility, nine to 14 plants per treatment were tested. Six control plants per treatment were likewise treated with sterile distilled water as inoculum. They made it possible to confirm that commercial samples of cultivars were free of anthracnose or other diseases. Apart from spraying inoculation where plants were already potted when inoculated, plants were transplanted after inoculation into 10-cm plastic pots containing a mixture of pasteurised sand and soil (1:3, v/v). Pots were placed in a greenhouse with a 16-h photoperiod using supplementary incandescent light and with 24 $\pm$ 6 °C day temperature and 18 $\pm$ 4 °C night temperature. High relative humidity, 90–95% rh, was maintained during five days after inoculation using a fog system. During all the experiments, plants were irrigated daily by spraying to allow the splash dispersal of conidia.

Disease response

The severity of disease symptoms was assessed on the following scale: 0 = no lesion, 0.5 = lesion just visible, 1.0 = a single developed lesion on petiole or foliage, 1.5 = two lesions, 2.0 = at least two leaves with expanded lesions, 2.5 = stunted plant but not wilted, 3.0 = beginning of wilting, 3.5 = two wilted leaves, 4 = almost all leaves wilted, 4.5 = all leaves wilted but slightly green, 5.0 = dead plant (Denoyes and Baudry, 1995). Notations were made two and four weeks after inoculation. Plants were rated into three classes of susceptibility according to their disease responses: class R for resistance reaction when notations were from 0 to 0.5, class I for intermediate reaction when notations were from 1 to 2.5 and class S for susceptible reaction when notations were from 3 to 5. The susceptibility of one cultivar can be expressed as the mean of disease responses or as the distribution of plant number or its percentage in each class.

Statistical analysis

Conformity of data to the main assumptions of the analysis of variance was checked, i.e., normality of distribution of the error terms, adequacy of the model and homogeneity of residual variances using the Bartlett test (Dagnelie, 1973). Analysis of variance and separation of treatment means with Tukey were then performed using SAS procedures (SAS Institute, Inc., Cary, NC). When assumptions of the analysis of variance were not made, the likelihood ratio chi-square test, G^2 test (or $2\hat{i}$ test) (Arbonnier, 1966) was performed on the distribution of plant number in three classes of susceptibility using SAS procedures. Statistical significance was considered at $P = 0.05$.

Results

Since symptoms were more consistent four weeks after inoculation than those obtained two weeks after inoculation, only the former were analysed. Whatever the transformation performed on data, the residual variances using the Bartlett test were not homogeneous in the two studies – comparison of inoculation techniques and evaluation for resistance to 1267b. However, they were homogeneous in the study of evaluation for resistance to 494a.

Results of G^2 test performed for each cultivar to compare the six techniques are presented in Table 1. Results obtained with the susceptible cultivars, Elsanta and Valeta, and the intermediate one, Addie, lead to the classification of inoculation techniques into two groups whereas no difference between the techniques is observed in the resistant cultivars, Sequoia and Dover which did not show wilting. The first group included spot and spraying inoculation techniques which caused few lesions and occasional wilting. When Elsanta plants were inoculated by spraying, repeatability of results between the two experiments was not obtained and experiment 2 showed more severe symptoms. When plants were inoculated by techniques of the second group, i.e., drop, injection or dipping, wilt symptoms increased compared with the two previous techniques and the drop technique caused less severe symptoms than the others. Injection and particularly the dipping technique minimise the number of plants without symptoms for the susceptible and intermediate cultivars. Therefore, in order to screen for cultivar resistance to *C. acutatum* using cold stored plants, we chose the technique causing most symptoms on susceptible cultivars, that is to say, the dipping inoculation technique using an inoculum concentration adjusted to 2.10^6 conidia ml^{-1}.

Standard cultivars, Elsanta, Osogrande, Sequoia and Dover, inoculated in nearly each experiment showed similar results between experiments [data not shown]. Thus, all experiments were pooled for analyses. Results of the G^2 test for evaluating resistance of the 44 cultivars to strain 1267b are shown in Table 2. Cultivars were classified from very susceptible to very resistant and showed all intermediate degrees of susceptibility. Eleven cultivars, from Darstar to Tiobelle showed susceptibility similar to Elsanta having a high percentage of wilted plants (>70% of plants in class S) and no or few plants in class R or in class I. Cultivars which had similar behaviour to the two resistant ones, Sequoia and Dover, were classified from Gariguette and from Primella, respectively, to Pandora. Classes of susceptibility of these cultivars were mainly restricted to class R since classes S and I showed a low to zero percentage of plants with symptoms. Seventeen cultivars from Addie to Dorit were similar for susceptibility to Osogrande. For these cultivars, the fungus caused wilt symptoms but with a lower percentage than for the Elsanta group (<17% except for Addie with 31% of plants in class S). Plant percentages of classes R and I varied from 33% to 88% and from 8% to 50% respectively.

Results of inoculation with strain 494a are shown in Table 3. In order to compare the distribution of plant number in the three susceptibility classes and the mean of disease response, G^2 and Tukey tests were carried

Table 1. Distribution of disease reponses to *Colletotrichum acutatum* in three classes of susceptibility and mean of disease responses of five strawberry cultivars inoculated by six techniques using cold stored plants

Inoculation techniques	Experiment number	Cultivars: Elsanta Classes[x] R	I	S			Valeta Classes R	I	S			Addie Classes R	I	S			Sequoia Classes R	I	S			Dover Classes R	I	S		
Spot	1	8	0	2	1.05[y]	a[z]	7	1	2	1.00	a	9	0	1	0.70	a	10	0	0	0.00	a	10	0	0	0.00	a
Spraying	1	7	3	0	0.40	b	8	2	0	0.35	a	7	3	0	0.40	a	10	0	0	0.00	a	9	0	0	0.00	a
Spraying	2	4	6	0	1.15	c	5	5	0	0.50	ab	9	1	0	0.25	a	10	0	0	0.00	a	10	0	0	0.00	a
Drop	2	0	3	7	4.20	d	2	5	3	2.50	bc	2	4	4	2.85	b	8	2	0	0.40	a	10	0	0	0.00	a
Injection	1	0	0	10	5.00	d	0	1	9	4.35	c	1	1	8	4.10	b	9	1	0	0.20	a	10	0	0	0.00	a
Dipping (10^6)	1	0	0	10	4.75	d	0	2	8	4.35	c	0	1	9	4.75	b	10	0	0	0.00	a	10	0	0	0.00	a
Dipping (10^6)	2	0	0	10	5.00	d	0	2	8	4.35	c	0	2	7	4.10	b	10	0	0	0.10	a	10	0	0	0.00	a
Dipping (2.10^6)	2	0	0	9	5.00	d	0	1	9	4.70	c	0	0	10	5.00	b	9	0	0	0.10	a	10	0	0	0.00	a

[x] Plant number distribution in three classes of susceptibility: R, resistant class – I, intermediate class – S, susceptible class.
[y] Each datum is the mean of nine or ten disease responses.
[z] Values in each column with the same letter do not differ significantly according to the G^2 test performed on plant number distribution ($P \leq 0.05$).

out and gave similar results. Osogrande was the most susceptible and Dover, the most resistant. Nevertheless, all the seven cultivars including those resistant to 1267b, i.e. Sequoia and Dover, showed wilt symptoms. Compared with 1267b inoculation results which were obtained in the same experiments, symptoms were less severe on susceptible cultivars such as Elsanta whereas they were more severe on resistant cultivars, Sequoia and Dover. These results are in agreement with those obtained previously (Denoyes and Baudry, 1995).

Discussion

The spraying inoculation technique has been reported for evaluating resistance to *C. acutatum* on cold stored plants (Simpson et al., 1994, Faedi et al., 1991), on seedlings (Smith et al., 1990; Gupton, Smith, 1991) or on dormant plants (Smith and Black, 1987). Nevertheless, in our test conditions, this technique, carried out on cold stored plants, gave inconsistent disease development and, therefore, was not usefull to screen for resistance even if the two susceptible cultivars were separated from the two resistant ones in experiment 2. Dipping inoculation technique using a conidial suspension adjusted to 2.10^6 conidia ml^{-1} was effective for screening for resistance to *C. acutatum* on cold stored plants and to minimise plants escaping inoculation. The slight dwarfing observed on cold stored plants inoculated by dipping compared to control plants was related to the weak issue of new roots. These old roots showed lesions caused by *C. acutatum* brought with the dipping inoculation. These results are in agreement with results obtained by Batta (1991) who described lesions on roots when plants were grown in soil infested with *C. acutatum*. Presence of *C. acutatum* in the crown with the dipping or injection techniques did not prevent the crown rot resistance from being expressed. However, previous studies using *C. fragariae* as inoculum advise against introducing it in the crown (Delp, Milholland, 1980). These differences in the expression of resistance could be due to the variability in pathogenicity between strains of *C. acutatum* and *C. fragariae*.

Variability of disease response according to isolates belonging to *C. acutatum* was obvious when plants were inoculated with 1267b and 494a separately. When 1267b was inoculated, twelve cultivars (from Capitola to Pandora in Table 2) did not develop wilt symptoms within 4 wk of inoculation and can be classified as very resistant to this strain. Thus, this resistance is present in cultivars bred in Europe such as Mamie and Pandora although breeding programmes for resistance to this fungus have not been carried out. When 494a was inoculated, all cultivars showed wilt symptoms, even cultivars resistant to 1267b, Sequoia and Dover. Variability of disease response according to the isolate also exists between our results and those reported in papers or in nursury beds. For example, Earlybelle was ranked as resistant in our test but intermediate when inoculated with *C. fragariae* (Delp, Milholland, 1981). Furthermore, in nursery beds, the wilting of Chandler

Table 2. Distribution of disease responses in three classes of susceptibility and means of disease reponses recorded 28 days after inoculation with *Colletotrichum acutatum* (strain 1267b) using the dipping technique on 44 strawberry cultivars

Cultivars[w]	Number of tested plants	Classes[x]			G^2 text ($\alpha = 5\%$)[y]	Means[z]
		R	I	S		
Darstar	24	0	0	100	a	5.0
Tustin	24	0	0	100	a	5.0
Hecker	24	0	0	100	a	4.9
Darestival	36	3	0	97	ab	4.9
Earliglow	36	0	8	92	abc	4.8
Maraline	24	0	8	92	abc	4.7
Fern	22	0	9	91	abc	4.7
Elsanta	*141*	*1*	*6*	*93*	*abc*	*4.7*
Pajaro	24	0	12	87	abc	4.6
Santana	24	0	17	83	abc	4.3
Kouril	24	12	8	80	bc	4.0
Tiobelle	24	4	25	71	c	3.8
Hokowase	23	26	35	39	d	2.5
Maxim	19	21	47	32	d	2.3
Addie	23	39	30	31	de	2.0
Cornwallis	24	33	50	17	de	1.6
Rosanne	35	46	43	11	def	1.2
Seascape	52	62	25	13	efg	1.0
Osogrande	*102*	*59*	*29*	*12*	*efg*	*1.0*
Arking	58	71	14	15	fgh	1.0
Darline	36	67	22	11	f-i	0.9
Jesco	23	61	26	13	f-j	0.8
Darlibelle	38	68	24	8	f-j	0.8
Chandler	44	75	14	11	f-j	0.8
Aïko	24	63	29	8	f-j	0.6
Allstar	50	78	16	6	f-j	0.6
Gariguette	49	82	10	8	f-k	0.6
Belrubi	34	79	15	6	f-k	0.5
Vicomtesse Hericart	38	79	16	5	f-k	0.5
Mara des Bois	24	83	13	4	f-k	0.4
Primella	24	88	8	4	f-l	0.4
Dorit	62	82	16	2	g-l	0.3
Capitola	38	76	24	0	h-l	0.3
Honeoye	50	80	20	0	h-l	0.3
Madame Moutot	24	87	13	0	h-m	0.2
Douglas	50	86	14	0	i-m	0.2
Parker	24	96	4	0	j-m	0.2
Sequoia	*110*	*95*	*5*	*0*	*klm*	*0.1*
Precosanna	24	96	4	0	lm	0.1
Mamie	24	96	4	0	lm	0.1
Dover	*89*	*96*	*4*	*0*	*lm*	*0.1*
Earlybelle	24	100	0	0	m	0.1
Pocahontas	24	100	0	0	m	0.0
Pandora	24	100	0	0	m	0.0

[w] Standard cultivars are italicized.

[x] The three susceptible classes are expressed as percentage of plant noted from 0 to 0.5 for class R, from 1 to 2.5 for class I and from 3 to 5 for class S.

[y] Values with the same letters do not differ significantly according to the G^2 test performed on plant number distribution ($P < 0.05$).

[z] Disease responses based on a scale of 0–5 represent the mean of at least two experiments.

Table 3. Distribution of disease responses in three classes of susceptibility and means of disease responses recorded 28 days after inoculation with *Colletotrichum acutatum* (strain 494a) using the dipping technique on seven strawberry cultivars

Cultivars[w]	Number of tested plants	Classes[x]			G^2 test ($\alpha = 5\%$)[y]	Means[z]	Tukey test ($\alpha = 5\%$)
		R	I	S			
Osogrande	*42*	*3*	*14*	*83*	*a*	*4.2*	*a*
Seascape	28	7	32	61	a	3.1	ab
Elsanta	*68*	*15*	*32*	*53*	*a*	*3.0*	*abc*
Addie	24	42	25	33	b	2.1	bcd
Sequoia	*40*	*48*	*17*	*35*	*b*	*1.9*	*bcd*
Darestival	26	46	31	23	b	1.8	cd
Dover	*68*	*49*	*35*	*16*	*b*	*1.2*	*d*

[w] Standard cultivars are italicized.
[x] The three susceptible classes are expressed as percentage of plants noted from 0 to 0.5 for class R, from 1 to 2.5 for class I and from 3 to 5 for class S.
[y] Values with the same letters do not differ significantly according to the G^2 test performed on plant number distribution ($P < 0.05$).
[z] Disease responses based on a scale of 0–5 represent the mean of at least two experiments.

recently observed in France could be due to a virulent pathogen although this cultivar has shown a low percentage of wilted plant when inoculated with 1267b in our experiment or a low disease severity rating in other experiment (Faedi, 1991). Nevertheless, other nursery observations agree with our results: Pajaro and Elsanta have shown wilting while Sequoia have not.

Fluctuation of disease response can occur within a cultivar and particularly within intermediate cultivars. Their disease responses were classified in the three classes of susceptibility instead of only the intermediate one. This might suggest a mechanism of resistance dependent on factors uncontrolled in our experiment. These remarks suggest that we have to test each genotype at least twice and use a large number of plants to evaluate the level of resistance. In addition, having a clear understanding of variability in pathogenicity of *Colletotrichum* species allows breeders to be sure of the stability of their resistant cultivars and to know the adaptability of their resistant cultivars developed in other regions or countries. Thus, successful screening will depend on including strains representative of the population found in commercial fields. This requires an effort to carry out a large-scale study on the pathogenicity variability of *Colletotrichum* species and on the genetic system of resistance.

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References

Arbonnier P (1966) L'analyse de l'information: Aperçu théorique et application à la loi multinomiale. Ann Sci For 31: 57–70

Batta Y (1991) L'anthracnose du fraisier due à *Colletotrichum acutatum*: Epidémiologie de la maladie et sensibilité de l'hôte. Thèse de doctorat. Inst. Nat. Agro., Paris, Grignon

Dagnelie P (1973) Théorie et méthodes statistiques. vol 2. 463 p. Les Presses Agronomiques de Gembloux, A.B.S.L. Gembloux. Belgique

Delp BR and Milholland RD (1980) Evaluating strawberry plants for resistance to *Colletotrichum fragariae*. Plant Dis 64: 1071–1073

Delp BR and Milholland RD (1981) Susceptibility of strawberry cultivars and related species to *Colletotrichum fragariae*. Plant Dis 65: 421–423

Denoyes B and Baudry A (1995) Species identification using morphological, and cultural characteristics and pathogenicity study of French *Colletotrichum* strains isolated from strawberry. Phytopathology 85: 53–57

Faedi W, Bagnara GL, Turci P, Winterbottom CQ and De Clauser R (1991) Valutazione della suscettibilità ad antracnosi di diversi cloni di fragola. In: Rosati P, Fraccaroli S and Febi A (eds) Nazionale sulla Fragola, Verona 8 Novembre 1991 (pp. 261–269) Società Orticola Italiana

Gupton CL and Smith BJ (1991) Inheritance of resistance to *Colletotrichum* species in strawberry. J Amer Soc Hort Sci 116: 724–727

Horn NL and Carver RG (1963) A new crown rot of strawberry plants caused by *Colletotrichum fragariae*. Phytopathology 53: 768–770

Maas JL and Howard CM (1985) Variation of several anthracnose fungi in virulence to strawberry and apple. Plant Dis 69: 164–166

Nourisseau JG (1986) Production of small fruit in France: Importance, main diseases and production of healthy plants. Acta Hort 186: 97–99

OEPP/EPPO (1994) Certification Scheme – Pathogen-tested strawberry – Bulletin OEPP/EPPO 24: 875–889

Perkins DD (1962) Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can J Microbiol 8: 591–594

Simpson DW, Winterbottom CQ, Bell JA and Maltoni ML (1994) Resistance to a single UK isolate of *Colletotrichum acutatum* in strawberry germplasm from Northern Europe. Euphytica 77: 161–164

Smith BJ and Spiers JM (1982) Evaluating techniques for screening strawberry seedlings for resistance to *Colletotrichum fragariae*. Plant Dis 66: 559–561

Smith BJ and Black LL (1987) Resistance to strawberry plants to *Colletotrichum fragariae* affected by environmental conditions. Plant Dis 71: 834–837

Smith BJ, Black LL and Galetta J (1990) Resistance to *Colletotrichum fragariae* in strawberry affected by seedling age and inoculation method. Plant Dis 74: 1016–1021