European Journal of Plant Pathology **108:** 565–571, 2002.

Evaluation of tests to determine resistance of *Zantedeschia* spp. (Araceae) to soft rot caused by *Erwinia carotovora* subsp. *carotovora*

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Accepted 14 May 2002

Key words: arum, calla lily, resistance, breeding, *Aestivae*

Abstract

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in *Zantedeschia* spp., particularly in cultivars from the section *Aestivae*. The disease can be partly controlled by cultural measures, but by combining cultural methods with resistant plant material a promising strategy for control of soft rot can be developed. No tests are available for resistance testing in breeding *Zantedeschia* spp. Therefore, three tests developed for use in potato breeding were adapted for use on eight cultivars of *Zantedeschia* spp. Variation was found in all three tests. Resistant control cultivar *Zantedeschia aethiopica* 'Crowborough' scored most resistant in all three tests. Within the section *Aestivae*, degrees of susceptibility were identified that were in agreement with each other and with field observations, indicating reliability of two of the methods in which tubers were used. The correlation coefficient of these two tests was high. A new non-destructive test method was developed for use on seedlings which involved immersion of leaf disks in a bacterial suspension. The percentage of decayed leaf area was a measure of resistance and results were in general agreement with the other tests. These methods will be useful for breeding for soft rot resistance and performing genetic analyses.

Introduction

Zantedeschia Sprengel (Araceae), also called 'calla lily' or 'arum lily', is a genus of about eight species in two sections, all from southern Africa (Letty, 1973; Singh et al., 1996). *Z. aethiopica* bears a rhizome and belongs to section *Zantedeschia*; hybrids with coloured flowers are developed from crosses of species from section *Aestivae*, mainly *Z. rehmannii*, *Z. elliotiana* and *Z. albomaculata* (Funnell, 1993; Singh et al., 1996). Species from this section all produce a root tuber as a storage organ (Robinson et al., 2000) and in contrast to *Z. aethiopica* require a dormancy period (Funnell, 1993).

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in section *Aestivae* of *Zantedeschia* and occurs worldwide (Corr, 1993; Funnell and MacKay 1999; Kuehny, 2000; Wright and Burge, 2000). This soilborne facultative anaerobic bacterium causes maceration and rotting of parenchymatous tissue of all plant organs, resulting in loss of the entire plant (Pérombelon and Kelman, 1980; Wright, 1998).

Plants become infected during storage or in the field, but infection does not necessarily result in soft rot, since the bacterium can be present latently (Funnell, 1993; Blom and Brown, 1999). Infected plants turn yellow, emit a foul smell and can be completely macerated resulting in death within a few days (Wright, 1998). Spread of bacteria takes place mainly by watering or by handling during tuber lifting, storage and planting. Soft rot symptoms can develop at any time during the growing cycle when conditions favourable for soft rot, such as high relative humidity occur, or when plants are under stress due to low soil aeration or high temperature (Funnell and MacKay, 1999; Wright and Burge, 2000).

Preventive soft rot control measures include irrigation, mulching and soil ventilation (Funnell, 1993; Funnell and MacKay, 1999; Wright and Burge, 2000). The bacteria are endemic in soil, making the disease difficult to control even with clean seed tubers (Pérombelon and Salmond, 1995; Funnell and MacKay, 1999). Increased calcium levels have been correlated positively with resistance to soft rot in calla lily, but no method gives full control of disease (Funnell and MacKay, 1999). Therefore, combining different cultural measures, including the use of resistant cultivars, is a promising approach in overcoming problems caused by the soft rot pathogen.

However, all *Aestivae* cultivars are susceptible to bacterial soft rot and no research is being conducted to determine variation in resistance within *Zantedeschia* spp. According to Funnell (1993) and Yao et al. (1995), *Z. aethiopica* is more tolerant to soft rot than *Aestivae* genotypes, but details were not provided. Incorporating resistance from *Z. aethiopica* into *Aestivae* genotypes is not possible due to major compatibility barriers (Yao et al., 1994; 1995). Therefore, sources of resistance must be located within the *Aestivae* gene pool and testing methods are required that can assess resistance levels in leaves and tubers. If possible sources of resistance are to be used in breeding for resistance, more knowledge is required on variation in and genetics of resistance. Therefore, methods for testing the resistance of clones must be developed, along with those individual seedlings. The latter requires special attention, since seedlings that are to be used in further crossing and evaluation must be tested by non-destructive methods.

Variation in resistance to *E. carotovora* has been studied in many crops (Allefs et al., 1995; Carputo et al., 1996; Darling et al., 2000; Ren et al., 2001). The potato–*Erwinia* complex, in particular, can be used as a basis for understanding the calla lily–*Erwinia* complex. Both crops are comparable in propagation and cultural methods. Both are mainly vegetatively propagated and the storage organ is used for over-wintering. In potato, this is a stolon-borne tuber, whereas *Z. aethiopica* bears a rhizome and plants from section *Aestivae* carry a root tuber (Robinson et al., 2000). Since *Aestivae* genotypes also carry tubers, it can be expected that methods developed for potato tubers could be used on *Zantedeschia* spp.

This paper describes the evaluation of four methods for determining resistance levels in tubers and leaves of *Zantedeschia* spp. The objectives were to evaluate methods previously used with potato to develop a new test method for *Aestivae* seedlings and to explore variation of resistance in *Zantedeschia* spp. to soft rot.

Materials and methods

Plant material

Plant material was obtained from commercial sources in The Netherlands. These included *Z. aethiopica* 'Crowborough' (section *Zantedeschia*), 'Best Gold', 'Black Magic', 'Galaxy', 'Pink Persuasion', 'Sensation', 'Treasure' and 'Florex Gold' (section *Aestivae*). For the tuber test, tubers from one-year-old plants were produced from *in vitro* propagated plants (T_1-tubers). Tubers used in the tuber slice test were from T_2-plants (two years from *in vitro* propagated plants). All tubers were stored at 9 °C at 70% relative humidity (RH) and treated for 10 min in 100 mg/l gibberellic acid (GA_3, Berelex, Bayer) before planting as is common practice to promote flowering (Funnell, 1993). Plants were grown from T_1-tubers in a greenhouse with day temperature of 20–30 °C and night temperatures of 15 °C.

Bacterial strain

E. carotovora subsp. *carotovora*, isolate PD 1784, isolated from an unknown *Zantedeschia* accession, was obtained from the Dutch Plant Protection Service and stored at −80 °C using 'Protect' beads (Technical Service Consultancy). Inocula were prepared from 48-h-old cultures in 'Lab-Lemco' Broth (Oxoid) and 86 mM NaCl (shaken at 100 rpm). Bacterial cultures were centrifuged for 10 min at 1800 *g* and the pellets resuspended in sterile tap water. The bacterial concentration was estimated using a haemocytometer.

Petiole test

The oldest leaves of plants were cut around flowering time with a knife disinfected in 80% ethanol. After discarding leaf blades, petioles were cut 20 cm from the top, washed three times in sterile water and surface-dried. Petioles were placed into 5 ml inoculum (1×10^5 cfu/ml) in plastic tubes (Ø = 2.0 cm) and incubated in an environmental chamber at 100% RH for five days. The length of healthy tissue (LH) was measured to the nearest 0.5 cm (modified from Bisht et al., 1993).

The experiment was done in two replicates using eight petioles per cultivar.

Tuber test

Whole T_1-tubers (8 per cultivar, not treated with GA_3) were disinfected by washing in tap water, immersing in 1% hypochlorite for 20 min and then again washing in tap water. Tubers were wounded by pushing a 200-µl pipette tip 3–5 mm into the base of the tuber (modified from Allefs et al. (1993) and Lojkowska and Kelman (1994)) and 20 µl inoculum (1×10^7 cfu/ml) was pipetted into the wound. Subsequently, tubers were incubated with the apical meristem pointed downwards in 100% RH, 20 °C until observation after six days. To measure the degree of resistance, the tubers were weighed before ($W1$) and after ($W2$) washing away infected tissue.

Tuber slice test

T_2-tubers were disinfected as described above. Ten slices, 7–9 mm thick, were cut longitudinally from three tubers using a clean knife. They were inoculated by placing a piece of conventional lab paper ($Ø = 5$ mm) soaked with 1×10^5 cfu/ml onto the middle of the cut surface. The slices were placed in a layer of water (approx. 1–2 mm deep), with the inoculated side up, to prevent drying of the cut surface. Incubation and analysis were done as in the tuber test, but the observations were done after two days.

Leaf disk test

Two young leaves of a newly sprouting plant were harvested just after folding of their leaf blades. Twelve disks ($Ø = 22$ mm) per leaf blade were made using a cork-borer and transferred to a 12-well plate in 5 ml inoculum (1×10^7 cfu/ml). Disks were kept immersed by constant pressure of a 1.5-ml eppendorf tube. Incubation was done in an environmental chamber (20 °C; 100% RH). Observations were made after three, four or six days of incubation, depending on level of symptoms. The percentage of decayed surface area (P) was visually estimated on a light-box. Every replicate included two leaves with 12 disks each.

The petiole and the tuber tests were carried out in duplicate during one season, the tuber slice test was done in duplicate in the second season, the leaf disk test was replicated at least three times in the first season, but most cultivars were measured in six replicates.

Statistical analyses

For the petiole and the leaf disk tests, differences between cultivars of healthy tissue (LH) and the percentage of macerated disk area (P), respectively, were estimated according to the iterative reweighted residual maximum likelihood algorithm (IRREML, assuming a binomial distribution using a logit link). This is a technique that can fit data sets to a Generalised Linear Mixed Model (GLMM). A GLMM is able to fit unbalanced data sets with random components of variance that are not normally distributed (Engel and Keen, 1994; Keen and Engel, 1998). In the tuber and tuber slice test, differences between cultivars for response variable $\sqrt{\{(W1 - W2)/W1\}}$ were estimated by ANOVA according to Haynes et al. (1997).

Spearman's rank correlation coefficients of the estimates of means of the cultivars in all test methods were calculated. Hereby, two calculations were made, one by inclusion and the second by exclusion of 'Crowborough'. This was done to assess whether 'Crowborough', which is the resistant control, biases the results. Moreover, the main interests were resistance differences within section *Aestivae* and use of the test methods in this group. All analyses were done using the statistical analysis software package Genstat 5, release 4.1 (Genstat 5 Committee, 1993).

Results

Four test methods were compared for measuring resistance levels to *Erwinia* in *Zantedeschia* spp. (Table 1).

Petiole test

'Crowborough' had the greatest length of healthy petiole tissue at 18.5 cm (see Table 1) which suggested a resistant phenotype. Cultivars 'Best Gold', 'Black Magic', 'Galaxy' and 'Pink Persuasion' had less healthy tissue ranging from 13.6 to 15.9 cm. Cultivars 'Sensation', 'Treasure' and 'Florex Gold' had little healthy tissue (susceptible phenotype) ranging from 6.9 to 10.9 cm which suggested that they were susceptible. However, due to high within-cultivar variance only 'Treasure' was significantly different (Table 1).

Table 1. Comparison of four test methods for estimating resistance of several *Zantedeschia* cultivars to *Erwinia carotovora* subsp. *carotovora* PD 1784

Cultivar	Petiole test[1]		Tuber test[2]	Tuber slice test[2]	Leaf disk test[3]	
		LH				*P*
'Crowborough'	2.90 a	18.5	0.00 a	0.00 a	−3.55 a	6
'Pink Persuasion'	0.82 ab	13.6	0.11 b	0.16 b	0.58 bc	55
'Black Magic'	1.27 ab	15.3	0.25 b	0.17 b	0.33 bc	54
'Best Gold'	1.33 ab	15.6	0.60 c	0.18 b	−0.28 b	40
'Galaxy'	1.68 ab	15.9	md[4]	0.20 b	0.91 bc	74
'Treasure'	−0.81 b	7.1	0.82 c	0.24 bc	0.92 bc	68
'Sensation'	−0.78 ab	6.9	0.73 c	0.31 c	−0.08 b	47
'Florex Gold'	0.31 ab	10.9	0.75 c	0.41 d	1.61 c	80
lsd[5]	3.00		0.11	0.07	1.55	

[1]Length of unmacerated petiole tissue on logit scale and in cm (LH).
[2]Relative weight of decayed tuber tissue: $\sqrt{\{(W1 - W2)/W1\}}$, where $W1$ represents the weight in grams of the tuber slice before and $W2$ after washing away macerated tissue.
[3]Percentage of decayed leaf disk area on logit scale and in % (P).
[4]Missing data.
[5]Lsd-values ($\alpha = 0.05$) are not applicable for LH and P, since these are not normally distributed.

Tuber test and tuber slice test

Using the tuber and the tuber slice test, respectively three and four groups could be distinguished (Table 1). As with the petiole test, 'Crowborough' had a resistant phenotype with no infected tuber tissue and all *Aestivae* cultivars were susceptible with a range of 0.11 to as much as 0.82 relative amount of infected tuber tissue. The most susceptible phenotypes in the petiole test, 'Sensation', 'Treasure' and 'Florex Gold' also were the more susceptible in these two tests (Table 1).

Leaf disk test

Decayed areas of the leaf disks were fully macerated. These were more transparent and visible as light green sectors in the disks when viewed on a light-box and disintegrated when touched.

Discrimination of cultivars after four days was slightly better than after three days (Table 2). After six days, only the resistant 'Crowborough' could be discriminated from all *Aestivae* cultivars. After both three- and four-day incubation, three *Aestivae* cultivars could be assigned to two levels of susceptibility. Cultivar 'Treasure' was known to be susceptible (Geerlings, pers. comm.), but after three days, 'Treasure' did not score as such. Therefore, a four-day incubation time was chosen as the best observation time for screening cultivars.

The four methods resulted in largely similar groups (Table 1). 'Crowborough' was least susceptible in all four test methods. Both 'Florex Gold' and 'Treasure' were most susceptible by all methods, while 'Pink Persuasion' and 'Black Magic' showed a lower susceptibility. Cultivars 'Best Gold', 'Sensation' and 'Galaxy' did not respond consistently. In the petiole and both tuber tests, 'Sensation' had a relatively susceptible phenotype, whereas in the leaf disk test, 'Sensation' was relatively resistant. 'Galaxy' was classed in the high susceptible group in the leaf disk test and in the low susceptible group using the petiole and the tuber slice tests. 'Best Gold' scored relatively resistant using the leaf disk test, but scored moderately susceptible using the tuber and petiole tests.

Including 'Crowborough' in the statistical evaluation resulted in higher correlation coefficients (Table 3).

Table 2. Means of percentage decayed leaf disk tissue (on logit scale) after three, four and six days after inoculation of seven *Zantedeschia* cultivars with *Erwinia carotovora* subsp. *carotovora* PD 1784

Cultivar	3 days	4 days	6 days
Crowborough	−3.82 a	−3.55 a	−3.32 a
Best Gold	md[1]	−0.28 b	1.63 b
Black Magic	−1.23 b	0.33 bc	1.13 b
Galaxy	−0.41 bc	0.91 bc	1.63 b
Pink Persuasion	−0.14 bc	0.58 bc	1.62 b
Sensation	−0.58 bc	−0.08 b	0.72 b
Treasure	−1.09 b	0.92 bc	2.34 b
Florex Gold	1.00 c	1.61 c	2.57 b
lsd ($\alpha = 0.05$)	1.60	1.55	2.75

[1]Missing data.

Table 3. Spearman's rank correlation coefficients of estimates of means from four test methods to measure resistance of seven *Zantedeschia* cultivars to *Erwinia carotovora* subsp. *carotovora*; including and excluding the results from resistant cultivar *Z. aethiopica* 'Crowborough' for determining correlation coefficients of results from six cultivars from section *Aestivae*

Test	Including 'Crowborough'			Excluding 'Crowborough'		
	Petiole	Tuber	Tuber slice	Petiole	Tuber	Tuber slice
Petiole	—	—	—	—	—	—
Tuber	0.82[1]	—	—	0.71	—	—
Tuber slice	0.67[1]	0.89[2]	—	0.50	0.83[1]	—
Leaf disk	0.55	0.64	0.62	0.32	0.43	0.43

[1,2]Significant at the 0.05 and 0.01 probability levels, respectively.

Only the correlation coefficient of the tuber and the tuber slice test was still significant after exclusion of 'Crowborough'. This indicated that the tests using tuber tissue are highly reproducible. The correlation between the leaf disk test and all other test results was not statistically significant.

Discussion

Four tests were compared for measuring resistance levels to soft rot. In general, all four test methods resulted in similar groups (Table 1), but there were considerable differences in variation and reproducibility. The tests using tubers gave the highest discrimination among the cultivars by revealing three and four significantly different groups in the tuber and tuber slice test, respectively (Table 1).

The high correlation ($r = 0.83$) between the results of *Aestivae* types in both tests using tuber tissue indicates high reproducibility (Table 3). However, only T_1-tubers were infected successfully after inoculation, i.e., resulting in a measurable amount of decay. T_2-tubers with a more irregular surface developed fewer symptoms (data not shown) and could have been latently infected. Latent infection of *Zantedeschia* spp. with *Erwinia* is known to occur in the field (Funnell, 1993). Hélias et al. (2000) also found latent infections after inoculation of potato plants.

In order to circumvent the establishment of latent infections, the tuber slice test was evaluated. All slices had a measurable amount of infected tissue after inoculating the cut surface of the tuber slice. Successful infection of irregularly shaped tubers apparently is dependent on the site of inoculation on the tuber. Bain and Pérombelon (1988) and Lojkowska and Kelman (1994) also found different results when inoculating different sites on the potato tuber. This could be related to an altered calcium concentration or cell wall content at the inoculation site (Pagel and Heitefuss, 1989; Pérombelon and Salmond, 1995).

The cultivar rankings by the tuber and the tuber slice tests were very similar (Table 3), so these results were not dependent on the site of inoculation. Although Allefs et al. (1995) found similar results in potato, Bain and Pérombelon (1988) and Lojkowska and Kelman (1994) found that cultivars were ranked differently by inoculating different sites on potato tubers.

The reasons for the high within-cultivar variances and the low correlation coefficients of the petiole and the leaf disk test ($r = 0.32$) are not clear. Other studies on *Erwinia* resistance also noted low reproducibility (Lojkowska and Kelman, 1994; Schober and Vermeulen, 1999), but was not explained. It could be related to the complex machinery involved in initiation of pathogenesis by *E. carotovora*, as modelled by Mukherjee et al. (2000), but this has not been studied in relation to infection in the field.

The low correlation coefficient of *Aestivae* cultivar rankings in the petiole and the leaf disk test ($r = 0.32$) compared with the tuber and the tuber slice test ($r = 0.83$) indicates a low reproducibility of results using leaf material. However, the composition of susceptibility groups by all the tests is similar (Table 1). The correlation coefficients of the two tests using tubers and the tests using leaf material are also low (Table 3). Allefs et al. (1996) also found low correlation coefficients between results of a tuber and a stem resistance test in potato after inoculation with *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*. They interpreted this as being due to different components of resistance.

Neither the petiole nor tuber tests are applicable for screening seedlings, as they use too much tissue or are destructive, respectively. The leaf disk method is the only non-destructive method available for testing *Zantedeschia* seedlings, since each plant develops only one tuber and 4–6 small leaves in the first year and 2–10 bigger leaves the second. A large number of leaf disks were used in this study. In seven replicates, up to 180 leaf disks were tested for some cultivars. Such large amounts of tissue are not available from single seedlings, but were necessary to find significant differences. It can be expected that less leaf material is needed when genotypes to be analysed have different levels of resistance. Hence, the most resistant and the most susceptible seedlings can be selected. The leaf

disk test could be optimised by including more than two leaves per genotype per replicate and using parts of leaves instead of whole leaves. This is supported by preliminary results from testing wild accessions and seedlings of section *Aestivae* (data not shown).

Observations of Shibuya (1956), Brown (1988) and Geerlings (pers. comm.) support the composition of groups as found in this study. They stated that *Z. elliotiana* was the most susceptible from section *Aestivae*, followed by *Z. rehmannii* and *Z. albomaculata*. Similarly, *Z. elliotiana*-resembling 'Florex Gold' was found most susceptible and *Z. rehmannii*-resembling 'Pink Persuasion' the least susceptible of the *Aestivae* cultivars in this paper. This indicates that there is agreement between field experiences and results obtained in our disease tests.

For selecting soft rot resistance during breeding of *Aestivae* genotypes, it is recommended to pre-screen seedlings using the leaf disk test and to screen subsequent clones at a later stage of the breeding programme using the tuber slice test. The pre-screen test can differentiate most susceptible and most resistant genotypes. The tuber slice test can be applied to estimate the level of resistance in selected clones more accurately. Hence, the way to breeding and genetic analyses has been opened. Variation in resistance within the genus *Zantedeschia* and genetics of resistance within section *Aestivae* are now being investigated.

Acknowledgements

The authors thank A. Hermsen for taking care of the plants in the greenhouse. The work of M.Q.M. van Paassen and H.-Y. Cho in performing disease tests and of Dr M.M.W.B. Hendriks in passing on statistical knowledge is highly appreciated. Prof Dr. P. Stam, Dr P. Lindhout and Dr R. Voorrips are thanked for critically reviewing the manuscript and Mr R. Geerlings for sharing field observations. This research was supported by the Dutch government, the Dutch Commodity Board for Horticultural Products (PT) and six companies.

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