Evaluation of a Cider Apple Germplasm Collection of Local Cultivars from Spain for Resistance to Fire Blight (*Erwinia amylovora*) Using a Combination of Inoculation Assays on Leaves and Shoots

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Abstract. Fire blight, caused by the bacterium *Erwinia amylovora*, is among the three most important diseases of apple. A major effective method for its integrated management is the reduction of the susceptibility of the host. Cider apple production in Spain is based on local apple cultivars with minimum crop management and phytosanitary control. After the entry of fire blight in Spain, the selection and planting of cultivars with low susceptibility to this disease has thus become of paramount importance. In consequence, and as part of a wider characterization effort, we undertook the evaluation of an apple germplasm collection of local apple cultivars from Spain for susceptibility to fire blight. Because of the quarantine status of *E. amylovora* in Europe, we evaluated the use of a detached leaf inoculation assay in combination with a traditional shoot inoculation assay to reduce the amount of plant material to evaluate and to minimize pathogen manipulation. Comparison of the susceptibility values for 78 apple cultivars indicated a low but significant correlation (*r* = 0.56; α = 0.01) between the leaf and shoot inoculation methods. Although the detached leaf assay was not reliable for the direct selection of cultivars with low susceptibility, it was useful to optimize resources and limit the potential dispersal of the pathogen by allowing the exclusion of medium and highly susceptible cultivars from further evaluation. Shoot inoculation of 103 apple cultivars allowed the identification of 48 cultivars with high levels of resistance to fire blight, which could serve as starting material both for apple production and for breeding programs.

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A collection of more than 250 cider and table apple cultivars originating from northern Spain has been established and maintained by the Instituto Técnico de Gestión Agrícola (ITGA; Navarre) and is being characterized (Itoiz and Royo, 2003; Lizar, 1996; Lizar et al., 2008). This germplasm collection could be exploited in the future as a source for resistance against fire blight and other diseases, either in rootstock or scion breeding programs, or as choice cultivars for cider production. We have thus undertaken the evaluation of this collection for resistance to diseases starting with the evaluation of their susceptibility to fire blight. Because of the quarantine status of *E. amylovora* in Spain, manipulation of the bacterium and inoculation of plant material had to be done under a very strict security protocol to avoid dispersion of the pathogen. We therefore tested the use of a leaf inoculation protocol to reduce the amount of plant material to evaluate, and the results of this assay were compared with the susceptibility ratings estimated using an inoculation assay with young shoots.

**Materials and Methods**

**Bacterial strains**

*E. amylovora* strains (Table 1) were generally propagated using King’s medium B (King et al., 1954) at 28 °C and were stored in this medium with 20% glycerol at −80 °C for long-term conservation.

**Plant inoculation**

Inoculation of detached leaves. Young leaves, 2 to 4 cm in lamina length, were collected during April and May from approximately 15- to 20-year-old trees located in an ITGA experimental orchard at Sartaguda (Navarre, Spain). Leaves were maintained refrigerated under high humidity until inoculation, which was carried out within 4 h from collection. Twenty-one leaves (seven leaves from each of three trees) were inoculated per cultivar; each set of seven leaves was considered a repetition because variation was larger between trees than among leaves from the same tree (data not shown). Inoculation was done essentially as described by Donovan (1991) with some modifications as follows. Leaves were surface-disinfected by immersion for 30 seconds in 1% household bleach and rinsed thoroughly three times, 1 min each, with sterile distilled water. Then, leaves were deposited over moist sterile filter paper in plastic boxes with a lid and 10 mL of a suspension of strain UPN500 (10^6 cfu mL^-1) was deposited on a fresh incision made in the midrib with a scalpel. Bacterial suspensions used for inoculation were obtained from cultures grown overnight at 28 °C on King’s medium B, which always were started from an original set of vials preserved at −80 °C in 20% glycerol. Cells were scraped from the agar surface and suspended in sterile distilled water adjusting the suspension to 10^6 cfu mL^-1 by dilution using a previously prepared viable-absorbance relationship. After 5 d in a growth chamber at 20 °C with a 16-h light (20000 lx) photoperiod, severity was estimated using a procedure modified from that of Duron et al. (1987). Briefly, progression of necrosis was evaluated using an arbitrary scale: 0 = no necrosis; 1 = necrosis limited to the inoculation point; 2 = necrosis advancing into the midrib; 3 = necrosis reaching the lateral veins; and 4 = necrosis over the whole leaf, reaching the end of the lateral veins (Fig. 1). These values were used to compute a necrosis severity index (NSI) using the formula: NSI = [(1 × n0) + (2 × n2) + (3 × n3) + (4 × n4)/N] × 100, where n0 is the number of leaves on each category in the scale to evaluate the progression of necrosis and N is the total number of leaves inoculated per repetition. For comparison with the inoculation assay on shoots, the NSI values for the detached leaves assay were transformed to an integer index value because this improved the correlation between the detached leaves and the shoot assays. The index transforms NSI percentage intervals into a 0- to 9-point scale as follows: 0 = 0%; 1 = 1% to 3%; 2 = 4% to 6%; 3 = 7% to 12%; 4 = 13% to 25%; 5 = 26% to 50%; 6 = 51% to 75%; 7 = 76% to 88%; 8 = 89% to 99%; and 9 = 100%. The NSI values of the different cultivars evaluated by both the detached leaf and the shoot assays were compared using the linear regression and Pearson’s correlation method.

**Shoot inoculation.**

Inoculations were carried out essentially as described by Norelli et al. (1988) by inoculating young shoots by cutting the youngest leaf with a scissors dipped in a pathogen suspension; this method performs better than others and results in optimal conditions in high disease severity levels (Ruz et al., 2008). To evaluate the susceptibility to *E. amylovora*, shoots were grafted on M.9 rootstock toward the end of August and kept in 2-L pots in the field; ≈3 weeks before inoculation, toward the beginning of April, they were moved to a greenhouse and then transferred to a growth chamber at 20 °C, 70% relative humidity, and a 16/8 h light/dark photoperiod (20,000 lx) for 1 week before inoculation. Five to eight plants were assayed per cultivar, and one to three actively growing, 20 to 30 cm long shoots per plant were inoculated by cutting the youngest leaf with a scissors dipped into a 10^{-6} cfu mL^-1 suspension of strain UPN500 prepared as indicated for the inoculation of detached leaves. As a result of the short length of the shoots, progression of the disease was estimated using an average NSI for each cultivar (Duron et al., 1987), which was recorded after incubating the plants for 12 d in a growth chamber. Before a one-way analysis of variance and a Student-Newman-Keuls test to determine differences in means (P = 0.05), the NSI values were transformed to arcsin [(√ NSI) × 0.01] × 57.2958; this last figure was used in the transformation to convert radians into degrees. According to their NSI, cultivars were classified into three arbitrary susceptibility groups (Le Lézec et al., 1997): low susceptible (0% to 40%), moderately susceptible (41% to 60%), and highly susceptible (61% to 100%). Additionally, the virulence of diverse *E. amylovora* strains was evaluated on shoots of 1-year-old self-rooted apple plants of cv. Gezamiña, maintained in 5-L pots, which were inoculated and rated as described previously.

**Results**

**Vulnerance of *E. amylovora* strains.** We were compelled to use a local isolate of the pathogen to test plant susceptibility and, in
consequence, we evaluated the virulence on apple of a collection of 14 strains of *E. amylovora* (Table 1), including six strains isolated in Spain. NSI values indicated that the strains showed significantly different degrees of virulence to apple (Table 2). Strain UPN500, isolated in Spain, was highly virulent to pear fruits (Cabreaga and Montesinos, 2005) and apple (Table 2) and was therefore chosen for further plant testing.

**Susceptibility of cultivars in the detached leaves assay.** Although the collection comprised more than 200 cultivars, we could evaluate only 233 cultivars using the detached leaves assay. In the more susceptible cultivars, symptoms appeared 2 to 3 d after inoculation and necrosis rapidly progressed through the midrib, the lateral veins, and the lamina, eventually covering the complete leaf by Day 5; bacterial exudates oozed out of some of these leaves. The mean NSI values for more than one-third of the cultivars were in the ranges of 21% to 40% and 41% to 60%, and only 6% of the cultivars scored in the extreme intervals (Table 3). We observed the variability typically associated with this type of assay with a SE of the mean of 5.4% for the NSI values, although it was lower than 5.5 for 34.7% of the NSI values.

**Susceptibility of cultivars in inoculated shoots.** As a result of operational limitations, mostly of space for inoculation under containment conditions, we only evaluated 103 local cultivars by shoot inoculation, which included the 40 cultivars with the lowest NSI in the detached leaves assay (up to 34%) plus 63 cultivars that were of interest for the local cider growers because of their pomological characteristics (B. Lizar, personal communication). In general, symptoms appeared 3 to 4 d later, and NSI values were recorded 12 d after inoculation. The 103 cultivars showed differential susceptibility to *E. amylovora*, displaying NSI values between 5% and 98% (Table 4) that allowed their classification into three arbitrary susceptibility groups. Forty-eight cultivars were included in the low susceptibility group, whereas 27 were considered moderately susceptible and 28 cultivars as highly susceptible. ‘Granny Smith’ was used as a positive control and, as expected (van der Zwet and Beer, 1995), was included in the highly susceptible group (Table 4). Variability was low, as expected for this kind of assay, and the analysis of variance showed significant differences of susceptibility to *E. amylovora* among the cultivars (Table 4).

A total of 78 cultivars were evaluated by both the detached leaf and the shoot assays and the resulting regression line ($y = 0.0171x + 3.6738$) had a low but significant correlation coefficient of $r = 0.56$, with $\alpha = 0.01$ (Fig. 2).

### Discussion

The adequate use of plant resistance is one of the more efficient, cheap, and environmentally sound control methods for fire blight; however, the majority of the most successful apple cultivars and rootstocks are highly susceptible to this disease, which has prompted the deployment of diverse strategies to incorporate the use of host resistance into fire

### Table 2. Virulence of different *Erwinia amylovora* strains on apple cv. Gezamihá

| Strain          | NSI ± sd | Classification |
|-----------------|----------|----------------|
| CFPB1430        | 87.8 ± 1.6 | A              |
| NCPPB3159       | 87.8 ± 1.6 | A              |
| UPN546          | 85.7 ± 2.5  | A              |
| EPS101          | 84.1 ± 3.0  | A              |
| NCPPB500       | 84.1 ± 3.7  | A              |
| NCPPB3548       | 81.3 ± 2.5  | A              |
| SV4576          | 80.7 ± 2.8  | A              |
| NCPPB683       | 80.5 ± 2.2  | A              |
| UPN529         | 79.2 ± 1.6  | A              |
| Es273          | 76.0 ± 1.9  | AB             |
| IVA1614-2       | 70.1 ± 2.0  | B              |
| OMP-B01185     | 57.8 ± 3.0  | C              |
| NCPPB595       | 53.9 ± 2.2  | C              |
| NCPPB311       | 28.1 ± 1.5  | D              |

*Mean necrosis severity index (from eight inoculated plants) ± sd.*

*The virulence level of strains classified in the same category are not significantly different according to one-way analysis of variance and Student-Newmans-Keuls test ($\alpha = 0.05$). The virulence level of strains classified in the same category are not significantly different according to one-way analysis of variance and Student-Newmans-Keuls test ($\alpha = 0.05$). The virulence level of strains classified in the same category are not significantly different according to one-way analysis of variance and Student-Newmans-Keuls test ($\alpha = 0.05$).

### Table 3. Distribution of local apple cultivars according to their susceptibility to *Erwinia amylovora* in a detached leaf assay and in a young shoot assay.

| Susceptibility group | NSI ± SD | Detached leaf assay | Shoot assay |
|----------------------|----------|---------------------|-------------|
| Low susceptibility    | 0% to 20% | 8                    | 17          |
|                      | 21% to 40% | 89                   | 31          |
| Moderate susceptibility| 41% to 60% | 71                   | 27          |
| High susceptibility   | 61% to 80% | 59                   | 18          |
|                      | 81% to 100% | 6                   | 10          |
| Total                |          | 233                  | 103         |

*Intervals of necrosis severity index (NSI) values.*

### Table 4. Classification of 103 local apple cultivars according to their susceptibility to *Erwinia amylovora* UPN500 in a shoot inoculation assay.

| Susceptibility groups and cultivars | Interval of NSI ± sd | Significance level |
|------------------------------------|----------------------|-------------------|
| Low susceptibility                  | 5 ± 1.8–8 ± 2.1      | a                 |
| Moderate susceptibility             | 9 ± 2.8               | ab                |
| High susceptibility                 | 10 ± 2.9–11 ± 2.2   | abc               |
|                                     | 14 ± 2.2–18 ± 4.6   | bcd               |
|                                     | 21 ± 2.4             | cdefg             |
|                                     | 22 ± 3.3             | defghi            |
|                                     | 24 ± 3.5–25 ± 0.0   | defghij           |
|                                     | 27 ± 1.6–30 ± 3.3   | efghijkl          |
|                                     | 31 ± 2.4–32 ± 3.2   | fgijklmn          |
| Moderate susceptibility             | 33 ± 3.8–35 ± 5.7   | fgijklmm          |
|                                     | 36 ± 3.7–40 ± 1.9   | fgijklmnop        |
| High susceptibility                 | 40 ± 4.0             | fgijklmnopqr      |
|                                     | 43 ± 3.1             | ghijklmnopqrstuv  |
|                                     | 45 ± 4.0             | ghijklmnopqrstuv  |
|                                     | 47 ± 2.0–50 ± 2.4   | ghijklmnopqrstuv  |
|                                     | 51 ± 8.1             | ghijklmnopqrstuv  |
|                                     | 53 ± 7.3             | ghijklmnopqrstuv  |
|                                     | 54 ± 3.7             | ghijklmnopqrstuv  |
|                                     | 55 ± 4.0             | ghijklmnopqrstuv  |
|                                     | 56 ± 3.1–56 ± 4.8   | ghijklmnopqrstuv  |
|                                     | 59 ± 3.9             | ghijklmnopqrstuv  |
|                                     | 60 ± 5.3             | ghijklmnopqrstuv  |
|                                     | 61 ± 3.7             | ghijklmnopqrstuv  |
|                                     | 64 ± 4.2–66 ± 3.6   | ghijklmnopqrstuv  |
|                                     | 68 ± 5.7–69 ± 5.3   | ghijklmnopqrstuv  |
|                                     | 73 ± 4.3–79 ± 6.1   | ghijklmnopqrstuv  |
|                                     | 83 ± 5.9–86 ± 6.1   | ghijklmnopqrstuv  |
|                                     | 89 ± 4.4–94 ± 3.5   | ghijklmnopqrstuv  |
|                                     | 98 ± 1.6             | ghijklmnopqrstuv  |

*Mean necrosis severity index (from the inoculation of five to eight plants per cultivar and one to three actively growing, 20- to 30-cm long shoots per plant) ± sd.*

*Same letters indicate no significant differences according to one-way analysis of variance and Student-Newmans-Keuls test ($\alpha = 0.05$). Cultivar used as highly susceptible control.*
fruit (Cabrefiga and Montesinos, 2005); a relevant exception was strain UPN546 that showed a very low virulence on pear fruit but was included in the group of highly virulent strains to apple. For evaluation we used strain UPN500, which was highly virulent to both pear fruit (Cabrefiga and Montesinos, 2005) and apple (Table 2) and was as virulent as other *E. amylovora* strains previously used for the evaluation of susceptibility to fire blight such as CFBP1430 or Ea273 (Cabrefiga and Montesinos, 2005; Laurent et al., 1989). Nevertheless, some apple cultivars are differentially susceptible to specific strains of *E. amylovora* (Cabrefiga and Montesinos, 2005), indicating the need to continue the evaluation of the cultivars with low susceptibility identified in this work using other bacterial strains, including those known to have exhibited differential aggressiveness. The high number of local cultivars showing low susceptibility to the disease is probably attributable, at least in part, to the fact that 40 of the 103 evaluated cultivars were selected among the less susceptible in the detached leaf assay; additionally, it is still possible that the germplasm collection is enriched in cultivars that have an increased resistance to diseases.

Because of the limitations for the manipulation of *E. amylovora* in Spain, and to speed up the identification of cultivars with low susceptibility, we applied a rapid susceptibility assay using detached leaves to narrow down the number of cultivars to be assayed using actively growing shoots, which is a more precise and reproducible method (Chevreau et al., 1998). The assay with detached leaves has the important advantages that it requires very little space for the inoculations, limiting the possibilities for pathogen escape, that a large number of cultivars can be assayed in a short period of time, and that there is a large supply of inexpensive material (i.e., leaves) to carry out the analyses. Previously, this type of analysis was successfully applied to evaluate the susceptibility of pear cultivars to *Pseudomonas syringae* (Moragrega et al., 2003), although it was reported to have limited reproducibility for the analysis of apple susceptibility to *E. amylovora* (Donovan, 1991; Donovan et al., 1994). In our hands, the detached leaf assay also showed large variability and low resolution, because scores for the leaf response to the pathogen were concentrated in less than half of the complete evaluation scale (Fig. 2), whereas scores in the shoot assay were distributed practically along the whole scale (Table 4; Fig. 2). On evaluation of the susceptibility of 78 apple cultivars by the detached leaf method and by inoculation of actively growing shoots, we found a low, but significant, correlation ($\rho = 0.56; \alpha = 0.01$) between the disease progression indices estimated for each cultivar with both methods (Fig. 2). However, the cultivars with low susceptibility to fire blight selected in the detached leaf assay (i.e., those having a score lower than 5, which is equivalent to an NSI of 40%) were distributed in the three susceptibility groups in the shoot assay; conversely, the large majority of cultivars showing a score higher than 5 in the detached leaf assay were later classified in the moderate or high susceptibility groups in the shoot assay (Fig. 2). Therefore, our results support previous observations that the detached leaf assay is not reliable for the direct selection of cultivars with low susceptibility to fire blight (Donovan, 1991; Donovan et al., 1994), although it was useful to identify some cultivars with a medium or high susceptibility to the pathogen. In our case, the use of the detached leaf assay was highly beneficial because the exclusion of these cultivars from shoot assays contributed to the optimization of resources and to limit the potential dispersal of the pathogen; these advantages would also be especially relevant in those situations in which it is necessary to evaluate a large number of cultivars such as during breeding for resistance.

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