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A LABORATORY METHOD FOR TESTING VIRULENCE IN ERWINIA AMYLOVORA STRAINS

BY

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ABSTRACT

A simple and easy laboratory technique is suggested for testing virulence of isolates of Erwinia amylovora, the causal of the fire-blight disease of pear and apple. In this work, detached apple or pear leaves were used in spring and summer months for testing virulence in the laboratory. In autumn and winter months, where desired young leaves from these host plants are not available, germinated pear seedlings could be used for this purpose. Seeds were germinated by the removal of the seed coats and directly planted in large trays containing moistened vermiculite. Suitable seedlings for inoculation could be produced within 2 weeks by this technique. These sources of plant material can now make testing virulence of E. amylovora isolates in laboratories, quite easy and satisfactory all the year round. Virulence of different strains could be determined within 48 hours-incubation period in inoculated wounded pear seedlings. Moderately virulent strains did not give any disease reaction at relatively low inoculum densities at this short period of incubation.

INTRODUCTION

Differentiation of virulent isolates of Erwinia amylovora was detected by various methods. Selective media were used for this purpose (Ark, 1937; Kado and Heskett, 1970; Miller and Schroth, 1972; and Crosse and Goodman, 1973). Testing virulence of the fire blight pathogen was usually done using succulent growing tissues (Crosse et al., 1972), thus making the timing of susceptible-tree growth important. This type of tissue is difficult to maintain in a large supply on a year-round basis, and the inoculated bacterium can possibly kill the trees which required one or more years to reach this stage. On this ground, several trials were made to find out a suitable source for determining virulence in suspected E. amylovora isolates. Layne (1964), suggested the use of cowpea (Vigna sinensis) for this purpose. Klement (1967), used the hypersensitive reaction of Nicotiana tabacum to differentiate pathogenic isolates of Pseudomonas spp. and Erwinia spp. Although both of these methods produce the desired results, yet they still require the use of

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potted plants. More recently, however, Pugashetti and Starr (1975) and Chatterjee and Starr (1976) were able to differentiate virulent strains of E. amylovora by inoculation into green pear fruits and pyracantha shoots. These two methods are extremely satisfactory, but the plant material is only available during 3 months or so during the growing season. A convenient laboratory method was suggested by Ritchie and Klos (1974), where they used germinating vernalized apple seedlings. However, vernalization process requires 3 months before the seeds could germinate.

In this work, a simple and rapid laboratory method for production of pear seedlings is suggested, together with the use of detached pear or apple leaves for testing the virulence of Erwinia amylovora strains all the year round.

MATERIALS AND METHODS

Seeds of Winter Nellis pear variety were soaked in running tap water for 48 hours, then partially surface-sterilized by soaking in sodium hypochlorite 0.5% for 20 minutes, then washed several times with sterile water. The seed coats were removed using a flamed scalpel, in order to break their dormancy. Care should be taken to avoid damage of the cotyledons. The seeds were then sown in large autoclavable plastic trays or large glass containers filled with sterile moistened vermiculite. Trays were covered with polyethylene sheets to maintain high moisture level around the germinated seeds and kept at room temperature (about 25°C.). Seeds began to germinate within 3-4 days and reached the size suitable for inoculation (10 cm long) after 2-3 weeks. Succulent growing shoots of apple or pear were excised from the trees growing in the orchard and thoroughly washed in a current of running tap water to remove most of dust debris and the adhering phyllospheric microflora. The shoots were let to dry on the lab-bench, then leaves were detached with the aid of flamed scissors and placed on the surface of dry, flame-sterilized glass slides in moistened dishes provided with a disk of filter paper in the bottom.

Several strains of the fire-blight pathogen (Erwinia amylovora), provided by M. P. Starr, Davis, California, U.S.A., were tested using either or all of the plant materials. Inocula were almost prepared from 24-hr-old cultures grown on Luria broth (g/l : 10 g Na Cl, 5 g yeast extract (Difco), 10 g L-tryptone). Inocula were prepared in water or 0.1 M phosphate buffer pH 7.0, to contain about 10^8 cells / ml. Bacterial suspension was injected in pear seedlings just below the cotyledons using a sterile disposable syringe with No. 25 gauge needle, and 0.01 ml of the bacterial suspension was injected per seedling. In other trials, the bacterial suspensions were inoculated by dropping of the bacterial suspension on the growing tip of the seedlings. In all cases, the boxes were covered after inoculation to maintain high moisture around the seedlings.

Inoculation of detached leaves was made by dropping 0.01 ml of the bacterial suspension on the leaf surface and a needle prick was made through the infection drop. All the plates were then kept at 30°C. To facilitate the introduction of bacteria into the leaf, particularly in older ones, they could be dispensed in the bacterial suspension and exposed to partial vacuum for 2 minutes, then transferred to the moistened dishes and incubated.

RESULTS

Development of the disease:

A. On pear seedlings:

Symptoms almost appear on individual seedlings after 2 days of inoculation and most seedlings showed different degrees of necrosis within 4 days. Copious bacterial droplets almost appeared on the necrosed area, depending on the strain used, on the fifth day. All seedlings collapsed within few days later. Non pathogenic strains, other than Erwinia amylovora (i.e. Escherichia coli) showed no symptoms at the inoculation site within one week (Fig. 1).

B. On detached apple and pear leaves:

Necrotic water-soaked lesions appeared at the site of inoculation after 2-5 days depending on the leaf age. Younger leaves produced faster reaction and necrotic lesions which almost spread to the adjacent healthy tissue when the incubation period was prolonged. Localized necrotic lesions appeared on older apple or pear leaves after 5 days and necrosis slowly progressed in the following days. Lesions on older leaves were irregular and do not exceed 7-8 mm in diameter after 10 days of incubation (Figs. 2 and 3).

Leaves infiltrated with the bacterial suspension almost showed necrotic lesions in the infiltrated area within 2-4 days of inoculation. When high moisture level was maintained around the inoculated leaves, bacterial exudates might cover the surface of the necrosed area few days later.

Effect of wounding:

Unwounded old leaves of apple or pear showed no symptoms even after 5 days of inoculation. However, in younger leaves small necrotic lesions appeared 4 days after inoculation but seemed to be localized at the inoculation site. Even though, some wounded leaves may show no necrosis particularly when the infection drop dried out in a relatively short period. Wounding is therefore essential for the introduction of the bacteria into the leaf tissues where it can multiply and produce disease symptoms. However, no symptoms appeared on leaves inoculated with non pathogenic bacteria (i.e. Escherichia coli).

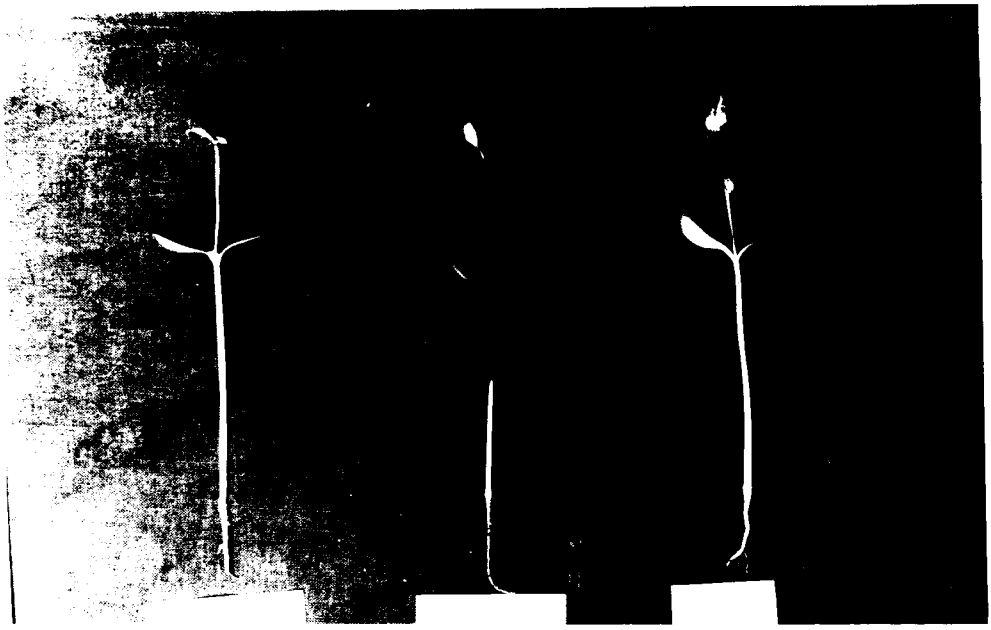

Fig. 1 : Pear seedlings inoculated with different bacterial cultures. a; on the left, healthy control inoculated with sterile water. b;in the middle, a seedling inoculated with Erwinia amylovora virulent strain showing typical symptoms of necrosis extending from the inoculation site. Notice bacterial droplets oozing from the necrosed area. c; a seedling showing no signs of ill-health, inoculated with a strain of Escherichia coli.

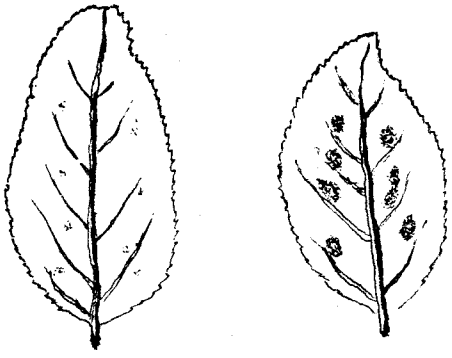

Fig. 2 : Detached apple leaf inoculated with a virulent strain of E.amylovora showing localized lesions on the right; control leaf on the left.

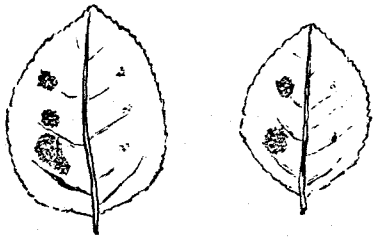

Fig. 3 : Detached pear leaves inoculated with a virulent strain of E.amylovora on the left halves showing necrotic lesions. Right halves represent controls.

Effect of inoculum density:

In this experiment, disease reaction of germinated pear seedlings was tested using different concentrations of the bacterial suspension. Three virulent strains were tested and serial dilutions from the initial stock of the bacterial suspension were prepared as described in Materials and Methods section. Inoculations were made in wounded seedlings by cutting 1 mm from the seedling-top leaves with the aid of flamed scissors and 0.02 ml of the particular suspension was dropped on the wounded tissues.

Results (Table 1) show that this technique could differentiate between the rate of virulence of different strains. In this test, the most virulent strain was EA 198 and the least one was EA 178 which could not produce any disease symptoms within 48 hours at inoculum density of 1.3×10^3 cells / ml (i.e. 10^{-6} of the initial stock density). Bacterial exudates developed more abundantly in seedlings inoculated with highly virulent strains within this short period of incubation. However, more bacterial droplets would develop on the necrosed tissues when the incubation period was prolonged. Higher inoculum densities resulted in more exudation in a relatively short period than lower density ones.

Table 1: Reaction of wounded pear seedlings to infection with different strains of Erwinia amylovora after 48 hours.

Serial dilutions from initial suspension	Disease reaction /1 EA 213/2	EA 178/2	EA 198/2
10^0	N^{3+} E^+	N^{3+} E^+	N^{3+} E^{2+}
10^{-2}	N^+ E^+	N^+ E^+	N^{2+} E^+
10^{-4}	N^+ E^-	N^+ E^-	N^+ E^+
10^{-6}	N^+ E^-	- -	N^+ E^-
Initial viable count	3.9×10^9	1.3×10^9	1.9×10^9

/1 Disease reactions were scored in the following manner:
Key ; N = necrosis E = exudates
- ; Do not produce necrosis or exudate after 48 hours.
+ ; Necrosis extending few mms to 1 cm, or tiny ooze droplets.
2^+ ; Necrosis extending to 1-2 cm, or many small ooze droplets.
3^+ ; Necrosis covering almost all the seedling tissues, or copious ooze at inoculation site and larger ooze droplets appearing on the necrosed tissues.

/2 Code number of strains provided from the ICPB (International Collection of Phytopathogenic Bacteria)

DISCUSSION

In this work, an easy and simple technique could be employed in plant pathology and bacteriology laboratories as it needs little space and can be provided at any time all the year round. During spring and summer months, apple or pear leaves could be detached from the growing trees in the orchard and used as a successful source for the determination of virulence of the suspected cultures of Erwinia amylovora. However, in autumn and winter months, young apple and pear leaves are not available and mostly gave inconsistent results. This could be interpreted on the basis of structural and other modifications that occur during maturation of leaves, and so a fewer infection sites will be available (Crosse et al., 1972). Another explanation for this phenomenon is the increasing effectiveness of the leaf defensive systems as they age (Ercolani, 1967 a and b).

It is suggested to use pear seedlings during winter months. The advantage of this technique than that used by Ritchie and Klos (1974) is that seedlings could be produced at any time needed without making a stock of vernalized seeds which needs about 3 months. Also, in this technique there is a chance to select healthy seeds for planting and therefore, unlike the vernalized seeds, about 100%, germination could be obtained from the germinated seeds. However, this technique might have the disadvantage of the difficulty to produce completely sterile seedlings for certain purposes, but for routine pathogenicity tests, it may be quite satisfactory, easy and rapid.

Injury of the leaves, especially the older ones was shown to be essential for uniform infections and for faster reaction. This agrees with the results of the earlier work of Brooks (1926) and Pierstoff (1931) and more recently by Crosse et al. (1972). However, the low rate of infection in non-injured leaves can possibly take place through natural openings as stomatal pores (Bauske, 1967; Lewis and Goodman, 1965; and Crosse et al., 1972). The rate of infection is related to the tension in the water conducting system and will tend to be low in high relative humidity, the condition in the already used moist inoculation chambers. Under similar conditions of high soil moisture and low transpiration activity, cherry leaf scars were found to be less susceptible to infection by Pseudomonas morsprunorum (Crosse, 1957).

Rate of virulence could be determined by short period incubation (48 hrs.) of inoculated wounded pear seedlings. Inoculum density was found to have a profound effect on disease reaction. Exudation was particularly affected denoting that exudation is a function of bacterial multiplication in the infected tissue. The more virulent strain can produce exudates at the lower inoculum densities, while the moderately virulent one cannot. This may show that the high rate of multiplication is a property of highly virulent strains (Chatterjee and Starr, 1976).

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طريقة معملية لاختبار القدرة المرضية لعزلات ايروينيا أميلوفورا

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اجرى هذا البحث لاختبار القدرة المرضية لعزلات الميكروب ايروينيا أميلوفورا المسبب لمرض اللفحة النارية فى التفاح والكمثرى على مدار السنه باتباع طريقة معملية سهله لاتحتاج الى حيز كبير . ففى خلال شهور الربيع والصيف أمكن اختبار القدرة المرضية بعدوى اوراق الكمثرى او التفاح الصغيره الغضه والمنزوعه من فروع الاشجار النامية واعطت اعراض التقرح المميزه للمرض . اما فى خلال شهور الخريف والشتاء حيث تكون هذه الاوراق غير متوفره على الاشجار فقد امكن حل هذه المشكلة بانبات بذور الكمثرى او التفاح عن طريق كسر طور السكون فيها بازالة أغلفه البذور ثم زراعتها فى أوانى تحتوى على فيرميكيوليت رطب وبذلك امكن الحصول على هذه البادرات فى خلال اسبوعين من الزراعة ، وامكن بحقن هذه البادرات بسلالات الميكروب المسبب للمرض ان تظهر الاعراض بالمقارنه بالحقن بميكروب اشيريشياكولاى الذى لم يعطى اى اعراض مرضية .

كما امكن التفريق بين القدره المرضية لسلالات الميكروب بحقن بادرات الكمثرى وتحضينها لمدة ٤٨ ساعة على درجة ٣٠° م بعد جرح الاوراق الطرفيه لها ـ وقد اتضح من ذلك ان السلالات عاليه القدره المرضية يمكنها اعطاء أعراض تقرح مع انتاج افرازات ميكروبية بغزاره كما يمكنها اعطاء اعراض واضحة للمرض عند الحقن بمعلق بكتيرى منخفض التركيز ـ بينما السلالات متوسطة القدرة المرضية لاتعطى اى اعراض مرضيه عند التركيز المنخفض خلال فترة التحضين القصيرة المستخدمه فى التجربه .