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Leaf, stem and crown galls on perennial asters caused by *Agrobacterium tumefaciens* in Taiwan

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Abstract. Leaf, stem, and crown galls induced by *Agrobacterium tumefaciens* were observed on field grown perennial asters (*Aster* spp.). Plants of purple flowers were more susceptible to infection than white flowers. The occurrence ranged from 25% in white flowered and 90% in purple flowered plants. Galls also occurred on leaves wounded by insect bites or mechanical shearing. *Agrobacterium tumefaciens* was isolated from crown and leaf galls with a selective medium NASA. The bacterial isolate was identified as *A. tumefaciens* using the Biolog GN system. Inoculation of selected *A. tumefaciens* strains on *Kalanchoe pinnata* leaves resulted in gall formation 8~10 days afterward. Several other *A. tumefaciens* strains from different gall samples also caused gall formation 6~8 days after inoculating on the stems of tobacco, and tomato seedlings. Re-inoculating virulent strains by scissors onto healthy aster leaves also induced galls 10 to 12 days after cut-inoculation. Biochemical tests of most *Agrobacterium* strains from aster galls showed that they belong to biovar 1.

Keywords: *Agrobacterium tumefaciens*; *Aster* sp.; Biovar 1; Crown gall; Selective medium.

Introduction

Agrobacterium tumefaciens is the causal agent of crown gall formation on many dicot plants, including ornamental species (De Cleene and De Ley, 1976). The bacteria transfers a segment of DNA (T-DNA) from Ti plasmid to a host cell, which then integrates itself into the host genome (Kado, 1991). As a result, the host develops a gall at the site of infection.

Perennial asters (*Aster* spp.) with pink, purple, or white florets on a long inflorescence stem are produced as cut flowers year round in central and southern Taiwan. We recently observed gall formation in the field grown purple variety in the southern part of Taiwan. *Agrobacterium tumefaciens* strain IL2 (possibly biovar 1) isolated from aster was briefly reported previously (Haas et al., 1995). However, a full characterization of aster *Agrobacterium* strains has not been done. *Chrysanthemum*, which also belongs to Compositae, was also reported to be susceptible to *A. tumefaciens* infection, which produced leaf, stem, crown and root galls (Miller, 1975; Bush and Pueppke, 1991). The Ti plasmid of *Agrobacterium tumefaciens* from chrysanthemum isolate, Chry5, was recently characterized molecularly (Bush and Pueppke, 1991). The isolation, detection and identification of *A. tumefaciens* depend on the use of several methods, including selective media, Biolog software characterization, and amplification of specific virulence and hormone biosynthesis regions by polymerase chain reaction (Haas et al., 1995; Moore et al., 1988; Ponsonnet and Nesme, 1994; Sawada et al., 1995; Serfontein and Staphorst, 1994).

In this study, we report the characterization of *A. tumefaciens* isolated from aster galls by a pathogenicity test, growth on selective medium, biochemical utilization pattern, and re-inoculation of healthy host asters.

Materials and Methods

Isolation and Maintenance of Agrobacterium tumefaciens

Leaf and crown galls of field grown, purple, perennial asters were collected from two locations in Pingtung County. The surface of the galls were removed by a handy blade and sterilized in 100 ml of 10% commercial bleach containing 4 drops of Tween-20 for 20 min. A 30 sec ultrasonic vibration (Branson 2200) was applied at the beginning of sterilization. After sterilization, the galls were washed three times with sterile water. They were then finely chopped and immersed in sterile water for 3 h or overnight. One loopful of the gall extract was streaked onto the Clark's selective medium as described in

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Serfontein and Staphorst (1994). The medium contains nutrient broth, 50 mg/l sodium selenite, 250 mg/l cycloheximide, and 15 g/l Sigma agar, and was designated as NASA medium. The plates were incubated at 28°C for two days. Putative brick red colonies from NASA were streaked on the same medium to purify single colonies. The purified colonies were cultured on YM medium (0.04% yeast extract, 1% mannitol, 1.7 mM sodium chloride, 0.8 mM magnesium sulfate, and 2.2 mM dipotassium phosphate, pH 7.0, 1.5% Sigma agar) and stored at 4°C, or cultured in liquid YM medium overnight at 250 rpm and frozen at -80°C with 25% glycerol until use.

Diagnostic Tests and Carbon Utilization

Colonies confirmed to be Gram negative bacteria, according to the method of Suslow et al. (1982), were streaked on Tryptic soy agar (TSA medium: Bacto tryptone 15 g/l, Bacto Soytone 5 g/l, Sodium chloride 5 g/l, Difco-Bacto agar 15 g/l) and incubated at 28°C overnight. They were applied into a microtiter plate containing 95 substrates. The ability to catabolise different compounds was measured by an ELISA reader and compared by Biolog software (Biolog Inc., Hayward, CA, USA). Bacterial colonies were identified using the Biolog GN system according to manufacture's directions (Microlog 3.5, Biolog Inc., Hayward, CA, USA).

Diagnostic tests for physiological and biochemical characterizations were conducted according to Moore et al. (1988).

Pathogenicity Test

The bacteria slurry grown on the YM medium was scraped off with a sterile surgery scalpel and slash-inoculated on both sides of the upper leaf epidermis of kalanchoe, *Kalanchoe pinnata* (Minnemeyer et al., 1991). The inoculated plants were cultivated on a balcony outside the laboratory. A control slash was made without bacteria. Gall formation was scored two months after inoculation. Selected stains were also inoculated on the stems of tomato (*Lycopersicum esculentum* cv. Known-You 301) and tobacco seedlings (*Nicotiana tabacum* cv. Xanthi NC). Colonies were also applied onto the top surface of carrot slices after surface sterilization by immersion and shaking in diluted Clorox.

Re-inoculation of Agrobacterium to Aster Leaves

Healthy lateral shoots of purple aster without galls were collected from the field and rooted under mist conditions. Each leaf was cut twice at both sides from the margin to half of the blade. A pair of sterile scissors was dipped in overnight grown bacterial suspension and used to cut the leaves. Control leaves were cut with sterile scissors without bacteria. Challenged pot-grown asters were wrapped in a plastic bag for two days to retain high humidity. They were then grown in a greenhouse with ambient temperature during February 1997. Gall formation was scored after two weeks.

Results and Discussion

Perennial asters from two farmers' fields in southern Taiwan were found to have stem, crown, and leaf galls (Figure 1A) after the inflorescence stems were harvested during the spring of 1996. About 25~90% of the field grown plants were galled. The underground parts of some plants also had dark brown to gray large galls. Galls were more common during the spring and fall season in southern Taiwan. During the summer, galls became disintegraded. The source of the aster cuttings was probably cut flower stems imported long ago because the plant is not native to Taiwan. The bacteria likely was imported and spread by vegetative propagation of aster cuttings by farmers. Plants with pink and purple flowers are more susceptible while white flowered varieties are less susceptible and produce fewer and smaller galls. This indicates that the severity of gall formation in asters is cultivar dependent. When leaves of field or pot grown plants are attacked by insects, the injured areas also produce small galls.

We were able to isolate smooth, round colonies with dark red centers and light transparent rings in the margin on the NASA selective medium from different gall samples. Several colonies were further purified by streak-plating on solid YM medium. Analysis of several strains by the Biolog GN program suggested that most were *A. tumefaciens*. The similarity to *A. tumefaciens* ranges from 74.8~82.6%. Other biochemical tests using the standard protocol (Moore et al., 1988) confirmed that most isolated strains belong to biovar 1 (Table 1) when compared to the authentic strain of biovar 1 (Chry5), which was previously isolated from chrysanthemum (Bush and Pueppke, 1991). However, some tested strains did not fit typical metabolisms of biovar 1. These exceptional strains are currently under investigation and will be reported elsewhere.

When the purified strains were inoculated on the leaf surface of kalanchoe, signs of gall formation were observed after 8~10 days. This induced small galls on the wound sites after 2 to 3 weeks. The galls tended to show vertical, rather than horizontal, growth (Figure 1B). Another twenty-seven strains were further inoculated onto tobacco and tomato seedlings. Signs of gall formation were observed on tomato stems about four days after inoculation. The galls grew larger after two weeks (Figure 1C). Galls on tobacco (Figure 1D) were much smaller than those on tomato. Galls formed on tobacco stems about 8 days after inoculation. Tobacco seedlings infected with stem galls became very weak and died later. Small dense galls were visible on carrot slices about 5 days after inoculation (Figure 1E), indicating that *Agrobacterium* isolated from aster gall is more virulent than that from *Ficus* galls (Hseu et al., 1997).

Table 2 shows the results of inoculating 26 strains on tomato and tobacco stems. More than 50% of the strains isolated from NASA selective medium were able to induce galls on tobacco and tomato stems, suggesting that aster agrobacterium showed pathogenicity on both hosts. The

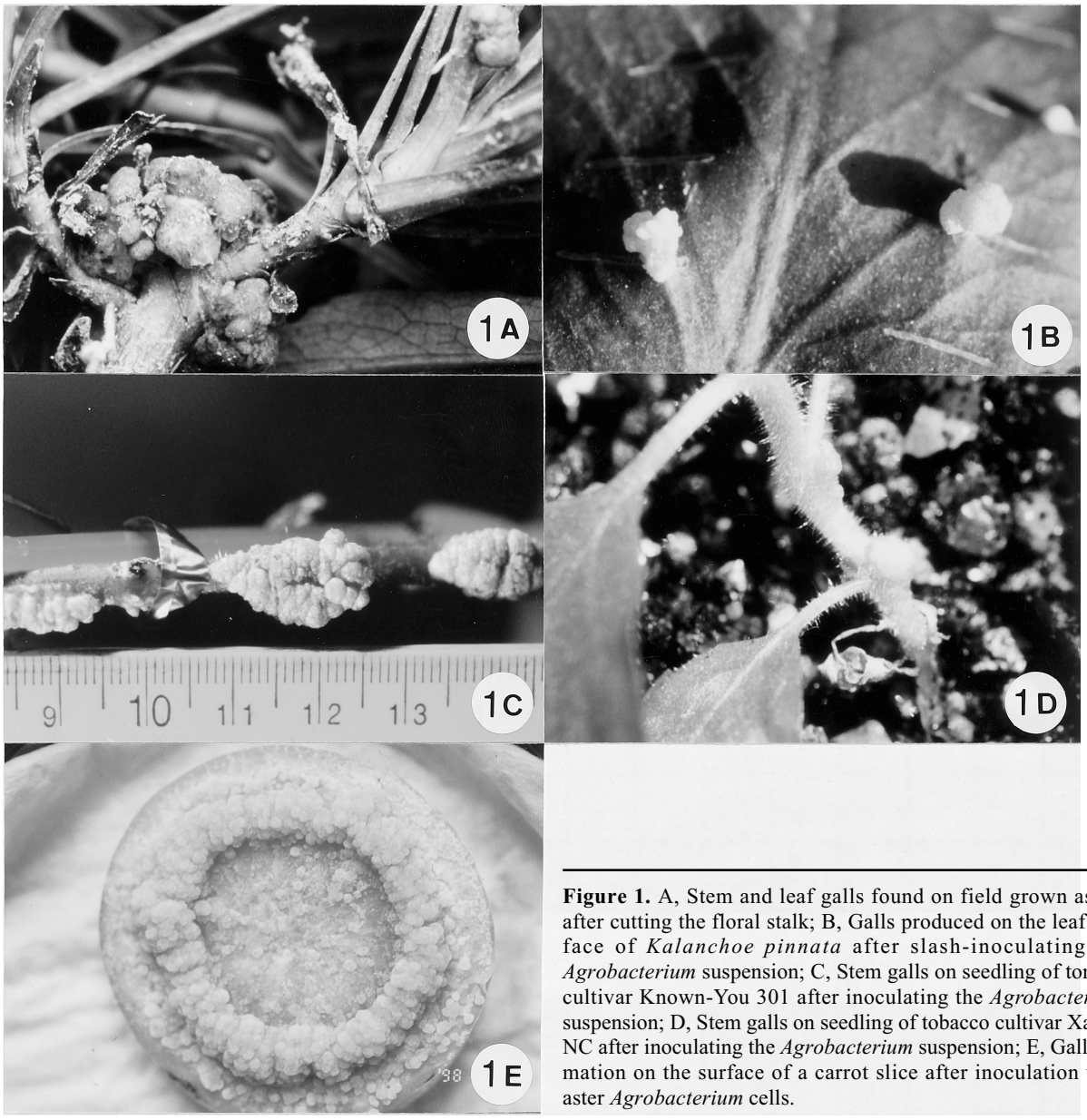

Figure 1. A, Stem and leaf galls found on field grown asters after cutting the floral stalk; B, Galls produced on the leaf surface of *Kalanchoe pinnata* after slash-inoculating the *Agrobacterium* suspension; C, Stem galls on seedling of tomato cultivar Known-You 301 after inoculating the *Agrobacterium* suspension; D, Stem galls on seedling of tobacco cultivar Xanthi NC after inoculating the *Agrobacterium* suspension; E, Gall formation on the surface of a carrot slice after inoculation with aster *Agrobacterium* cells.

selective NASA medium is therefore suitable for *Agrobacterium* isolated from aster galls.

When leaves of healthy aster plants were inoculated with 10 *Agrobacterium* strains, they all produced visible small galls after 10 to 12 days on the wound sites (Figure 2). The control leaves, on the other hand, did not show any symptoms of galls. Galls on the treated leaves tended to emerge from the veins, an indication of *Agrobacterium* infection in the tissues active in cell division.

We previously reported the isolation of *A. tumefaciens* from ornamental *Ficus microcarpa* trees (Hseu et al., 1997) in central and southern Taiwan. The occurrence of *Agrobacterium*-induced galls in Taiwan in perennial asters has not been recorded previously. This study is perhaps the first report of galls from field grown asters in Taiwan. The morphology of aster *Agrobacterium* is simi-

Figure 2. Gall formation 10 to 12 days after re-inoculation of aster *Agrobacterium* on aster leaf.

Table 1. The characterization of biovar of *Agrobacterium tumefaciens* from aster and chrysanthemum.

Diagnostic test	Biovar 1[a]	Chrysanthemum (Chry5 strain)[b]	Aster (4 strains)
3-Ketolactose production	+	+	+
Growth in 2% NaCl	+	+	+
Growth at 35°C	+	+	+
Action on litmus milk			
Alkaline	+	+	+
Acid	–	–	–
Acid from:			
Sucrose	+	+	+
Erythritol	–	–	–
Melezitose	+	+	+
Alkali from:			
Malonic acid	–	–	–
L-tartaric acid	–	–	–
Propionic acid	V	–	–
Mucic acid	–	–	–
Ferric ammonium citrate	+	+	+
L-tyrosine utilization	–	–	–
Citrate utilization	V	+	+

[a]Data from Moore et al., 1988.
[b]Chry5 was kindly provided by S.G. Pueppke as a reference for biovar 1.

Table 2. Gall formation in stems of tomato and tobacco inoculated with *Agrobacterium tumefaciens* strains isolated from aster.

Strain	Gall on tomato stem[a]	Gall on tobacco stem[b]
Aa-1	+	+
Aa-2	+	+
Aa-3	+	+
Aa-4	+	+
Aa-5	+	+
Aa-6	–	–
Aa-7	–	–
Aa-8	+	+
Aa-9	–	–
Aa-10	–	–
Aa-11	–	–
Aa-12	–	–
Aa-13	+	+
Aa-14	+	+
Aa-15	+	+
Aa-16	+	+
Aa-17	+	+
Aa-18	ND[c]	ND
Aa-19	+	+
Aa-20	–	–
Aa-21	+	–
Aa-22	+	+
Aa-23	ND	+
Aa-24	ND	+
Aa-25	–	ND
Aa-27	+	ND
Control	–	–

[a]Known-You cultivar 301.
[b]*Nicotiana tabacum* cv. Xanthi NC.
[c]ND, not determined.

lar to that of *F. microcarpa* when viewed under electron microscope (Hseu et al., 1997); both were rod-shaped peritrichous bacteria.

Since most asters are susceptible to *Agrobacterium*, it is desirable to produce healthy propagules free of the bacteria using meristem or leaf explant culture coupled with antibiotic treatment and other suitable control measures. One commercial aster supplier describes in its catalog its effort to eliminate the agrobacterium from stock plants in order to establish clean micropropagation materials (Danziger "Dan" Flower Farm Catalog, Israel, date unknown). Also, a method capable of detecting infected asters early, in the nursery or in the cut flowers, needs to be developed. Amplification of certain conserved regions of Ti plasmids by polymerase chain reaction has been suggested as a method of diagnosis (Haas et al., 1995; Ponsonnet and Nesme, 1994; Sachadyn and Kur, 1997; Sawada et al., 1995).

Since the isolated agrobacteria cells were able to induce galls on solanaceous crops such as tobacco and tomato, and on carrot slices, they supposedly contain tumor inducing genes for, e.g. auxin and cytokinin biosynthesis. They also cause the rapid growth of galls after inoculation, an indication of strong virulence on herbaceous plants. The *ipt* and *rolC* genes have been cloned from other agrobacteria strains/species and introduced into the genome of higher plants to modify their morphology (Brzobohaty et al., 1994; Michael and Spena, 1995). It is possible to isolate the same gene homologues from aster

agrobacteria that we reported here. Modified agrobacterium has also been used as a vector for plant genetic engineering, and we suggest that aster agrobacterium has potential as a tool to introduce foreign genes into important crop plants.

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台灣地區宿根紫苑莖部及葉片腫瘤是由細菌性癌腫病菌 (*Agrobacterium tumefaciens*) 所引起

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宿根紫苑爲一新興切花作物，有多種花色，其中兩個品種 '紫孔雀' 及 '白孔雀' 在 85 年春於屏東地區發現在修剪過的植株基部、根冠、葉片及切口處均長出大小不等的腫瘤，大者約可達直徑 5公分，有些植株受昆蟲咬食後之葉片會延著傷口長出小形腫瘤。不同品種間發生腫瘤的比率有差異，紫孔雀品種在田間約有 90% 以上的植株都發現產生腫瘤，白孔雀之發生率則較低，約 25%，最近又觀察到另一個新引入的白花品種部份植株也在莖部及葉片傷口產生較小腫瘤。紫孔雀腫瘤利用無菌水萃取並以 NASA培養基分離得到磚紅色之圓形菌落，以YM培養基純化之菌落爲白色、突起、光滑且爲圓形。經以 Biolog GN Microplate (Biolog, Inc., Hayward, CA, USA) 培養鑑定爲 *Agrobacterium tumefaciens*，當以生化檢定方法與標準 biovar 1 菌系 Chry5 比較時，顯示多數紫苑菌系均屬於同一 biovar。將細菌以 YM 或液體之 YM 培養基培養過夜後，以針刺或解剖刀接種於落地生根葉片、農友 301 番茄及煙草 (*Nicotiana tabacum* cv. Xanthi NC) 幼苗之葉片或莖部 (番茄及煙草)，番茄及煙草在接種後 6~8 天於接種處產生白色腫瘤，其寬度約 1~1.5 公分，而在落地生根葉片上之腫瘤約在 8~10 天才開始形成且較小，且呈柱狀或不規則圓球狀。將分離之菌落培養成懸浮液並以剪刀回接紫苑葉片，在 10~12 天後，於切口位置長出小瘤，對照組則沒有變化。由以上的試驗結果，證實造成 '紫孔雀' 腫瘤病之病原菌爲農桿癌腫病菌 *Agrobacterium tumefaciens* biovar 1。

關鍵詞：癌腫病菌；紫苑屬；生物型 1；腫瘤；選擇性培養基。