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ABSTRACT

Infected golden bush have water soaked to brown angular lesions which are often necrotic. This is often observed in young infected leaves. When severe, the lesions eventually drop off leaving a shot-hole appearance.

There were two isolated bacteria: one with yellow colonies and another with white colonies. The isolates were both Gram-negative rods and were motile but varied culturally and physiologically. The yellow bacterium exhibited characters typical of a *Xanthomonas campestris* pathovar while the white bacterium resembled *Pseudomonas.*

Inoculation of either bacterium did not result in symptoms even using various means of inoculation; rubbing of leaves, injection on stem and leaves and dipping of roots. However, dipping of leaves in a combined suspension of the two isolates resulted in an angular spot symptoms after 48 hours of incubation.

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INTRODUCTION

Duranta repens L. (locally called golden bush) is one of the genus of about 30 species of evergreen trees and shrubs. Although tropical America is the home of the golden bush (also known as pigeon berry, sky flower and golden dewdrop) (Brigham, 2001), it has been in the Philippines since 1880 (Steiner, 1986). It produces its starry blossoms throughout the year and its yellow-speckled to golden yellow leaves have made it quite popular as a hedge. The ease with which it is cultivated adds to its popularity and it is seen growing in most towns and cities in the Philippines.

The plant is attacked by a leaf spot characterized by angular lesions which are often necrotic and which eventually drop off leaving a shot-hole appearance in severe cases. This disease can be observed on most golden bush plantings. Initial microscopic observation of lesions points to a bacterial origin. Tangonan (1999) does not have any record of *Duranta* or its diseases in the Philippines.

It is therefore important to verify the etiology of this leaf spot and identify its causal bacterium. Knowledge of this causal bacterium will determine whether it poses a threat to other ornamentals and important crops here in Benguet.

The study was conducted to describe the symptoms of the angular leaf spot of golden bush, isolate and characterize the bacterium associated with the symptoms and verify the pathogenicity of isolates on golden bush.

The study was conducted from August 2005 to January 2006 at the Plant Pathology Department service laboratory, College of Agriculture, Benguet State University, La Trinidad, Benguet.

REVIEW OF LITERATURE

Host

Duranta repens L. locally known as golden bush belongs to family Verbenacea (Christman, S., 2003). The genus is named after Castor Durantes, an Italian botanist and doctor. Puneet (2004) posted the following blog regarding the taxonomy of *Duranta*.

Kingdom--------------------->Plantae – Plants

Subkingdom-------------->Tracheobionta – Vascular plants

Superdivision----------->Spermatophyta – Seed plants

Division---------------->Magnoliophyta – Flowering plants

Class----------------->Magnoliopsida – Dicotyledons

Subclass---------->Asteridae

Order----------->Lamiales

Family------->Verbenaceae – Verbena family

Genus------>*Duranta* L.

It is a shrub to a small tree, with slender erect or drooping, branches four to five meters tall. The whorled or opposite leaves are smallish, often toothed; and the 5 – petalled flowers, narrowing to a short tube, appears in delicate sprays from the upper leaf axils (Anonymous, 2001). The white to blue flowers are produced in clusters. Its corolla is formed into a tube and spreading into five limbs. Fruits are rounded yellow or orange and 1.5 cm across. The firm fleshy, orange or yellow berries are allegedly poisonous.

Duranta species are vigorous. They thrive in almost any kind of soil, in full sun. They can be trained to become small trees or kept cut back as shrubs; they make useful

hedges. Propagation is by cuttings although they are sometimes difficult to root, but the plant can be propagated from seed or by layering and marcoting at the beginning of the rainy season.

Diseases Caused by Bacteria

According to Agrios (1997), the most common types of bacterial diseases of plants are those that appear as spots of various sizes on leaves, stem, blossoms, and fruits. In some bacterial diseases, the spots continue to advance rapidly to produce blights in two ways. In severe infections, the spots may be numerous that destroy most of the plant surface and the plant appears blighted or the spots may be enlarged and coalesce, thus producing larges areas of dead plant tissue and blighted plants.

The spots are necrotic or roughly circular and in some cases surrounded by a yellowish halo. In dicotyledonous plants, large veins restrict the bacterial spots on some hosts and the spots appear angular. Agrios (1997) further cites that the bacteria enter the leaf through stomata, hydathodes and wounds. Certain insect such as beetles, aphids and white flies also act as vectors of the pathogen.

The International Society of Plant Protection (2001) records the presence of a pathovar of *Xanthomonas campestris* that is named *durantae*. From this name, it can be assumed that it was probably isolated from a *Duranta* species. The National Collection of Plant Pathogenic Bacteria (2005) confirms this information. The full record of the epithet is given by both references as *Xanthomonas campestris* pv. *durantae* (Srinivasan and Patel, 1957) Dye, 1978.

MATERIALS AND METHODS

A. Collection of Diseased Plants

Samples of infected golden bush was collected around La Trinidad and placed in transparent plastic bags and brought for diagnosis to the laboratory.

B. Description of Symptoms

Symptoms of the plants were described then sections from the infected tissue were studied under the microscope. All observations were recorded.

C. Identification of Isolated Bacteria

Isolation of associated bacteria. Isolation and culture of associated bacteria was done first on Potato Dextrose Peptone Agar (PDPA). The media was prepared following the standard procedure. The collected disease plant was washed in tap water to free them from dirt then leaf samples were washed in 1% sodium hypochlorite and three change of sterile distilled water then blotted on sterile tissue paper. Leaf samples were placed in 10 ml of sterile water and macerated with a flamed glass rod. A loopful of the suspension was streaked onto previously prepared PDPA plates and incubated at 27-30oc for two days. At least three well-separated, isolate single colonies were transferred into PDPA slants. Stocks cultures will be at 5 C.

Identification of isolated bacteria. The following tests were performed to determine the genera of the isolated bacteria.

1. Growth on various media [Appendix 1]

    a. Yeast Extract Dextrose $CACO_3$ Agar (YDCA)

    b. King's Medium B Agar (KMBA)

c. Nutrient Glucose Agar (NGA)

d. Sucrose Peptone Agar (SPA)

e. *Xanthomonas* Isolation Medium (XIM)

2. Gram stain and KOH test

3. Other tests. The procedures for the following tests are as outlined by Schaad (1998).

a. Growth and O/F metabolism. Basal medium (10 ml) was poured in 10 test tubes having a diameter of 13 cm. Two drops of bromthymol blue solution was dropped in each test tube and shaken to mix the suspension. Water agar prepared in a separate flask was used to cover the basal medium in the test tube. For each replication, the bacterial isolate was stabbed in 2 test tubes of basal medium after which one test tube was covered and the other uncovered. The set-up was incubated at 24 to 48 hours and change in color was observed and documented.

b. Gelatin Liquefaction test. Commercial gelatin (5g) was added in 1 liter distilled water and dissolved by steam heating. Ten ml was dispensed in 15 test tubes having a diameter of 13 cm. Sterilization was done for 12-15 minutes at 121°C and cooled immediately without slating. Each bacterial isolate grown 24 hours were stabbed into the medium. Incubated at 20 to 27^{0}C for 21 days. After 3,7,14 and 21 days, the test tubes was refrigerated for 30 minutes. If the medium flows readily as tube is gently tipped, the gelatin has been hydrolyzed and denotes a positive reaction from the test. The test is considered doubtful, if the medium moves a very slowly result as compared with non-inoculated medium.

c. Motility test. Sterile water (0.5-1.0 ml) was added to actively growing bacteria on nutrient agar slants. The slants were incubated overnight. A loopful of the bacterial suspension was taken from the bottom of the slant and placed on the underside of a cover slip. A depression slide was inverted over the drop of suspension on the slip. The slide and cover slip was turned over and the drop viewed under the microscope.

d. Starch hydrolysis test. The bacterium was streaked onto starch agar and the plates were then incubated for 2-7 days. The plates then were flooded with an iodine solution and any zones of clearing were recorded.

e. Other biochemical test as needed

D. Pathogenicity Test

Test plants. Clean (apparently disease-free) and young golden bush plants was obtained and kept in the greenhouse for pathogenicity test.

Inoculum preparation and inoculation. Suspension of isolated bacteria was prepared and standardized at 1×10^6 cfu/ml. Suspension was sprayed onto healthy plant leaves. The inoculated plant was covered with a plastic bag and incubated overnight in the laboratory. After overnight incubation, the plastic bag was removed and the inoculated plants were then brought to the greenhouse. Leaves were observed for symptom development. Symptoms observed were compared to the initial observations.

Re-isolation of causal organism. The causal organism from the inoculated test plants was re-isolated onto nutrient agar. Isolates thus obtained were compared to the original isolates.

Data Gathered

The following data were gathered:

1. Symptoms of the diseased plant caused by bacterial pathogen. The symptoms of the plant were observed and described.

2. Cultural, morphological and physiological characteristics of the isolates. The cultural, morphological, and physiological characteristics on the various media were observed. Specifically, the following colony characters were noted: size, shape, color, elevation, margins, and production of pigment on the media.

3. Pathogenicity test results. The symptoms produced by the inoculated bacterium was noted and then compared to the original symptoms observed.

4. Photodocumentation. All set-ups were photographed.

RESULTS AND DISCUSSION

Disease Symptoms

Infected golden bush has water-soaked to brown angular lesions which are often necrotic, this is often observed in young infected leaves. When symptoms are severe, the lesions eventually drop off leaving a shot-hole appearance (Plate 1).

Bacteria in the genera of *Pseudomonas* and *Xanthomonas* cause most bacterial spots and blights. These bacterial diseases appear as spots of various sizes on leaves, stems, blossoms and fruits. Sometimes, the spots continue to advance rapidly, eventually resulting in blights (Agrios, 1992).

Plate 1. Infected leaves of *Duranta repens*

Cultural Characteristics

A loopful of bacterial suspension was streaked on Nutrient Agar (NA) and incubated for 24 hours (Plate 2). Yellow and white colonies were observed on the plate. Isolated single colonies were transferred onto NA slant and served as stock culture.

Both bacteria were streaked on SPA, NGA, KMBA, YDCA and XIM. The yellow bacterium grew after 24 hours and produced yellow pigmentation on NGA and SPA (Plate 3). On YDCA and KMBA, the bacterium was observed after 48 hours and produced light yellow and dark yellow colonies, respectively. On the *Xanthomonas* Identification Medium, yellow colonies were observed only after 4 days of incubation.

Plate 2. The bacterium on nutrient agar (24 HAI)

These characteristics coincide with the description of the genus *Xanthomonas.* Schaad and Stall (1998) described *Xanthomonas* to have the following characteristics: colonies are mucoid, convex and shiny on NGA and YDC agar and produce unique yellow membrane bound pigments that are non-H_2O soluble. They also added that bacteria obtained from dried material may take up to a week to form single colonies. Dianese and Schaad (1982) had mentioned that most *Xanthomonas* strains form yellow water soluble pigments (xanthomodins) when cultivated on the common growth media such as GYCA, LPGA and NA.

The white isolate produced light yellow colonies on NGA and SPA after 24 hours of incubation (Plate 3). The bacterium also produces light yellow colonies on KMBA

and white colonies on YDCA after 48 hours of incubation. It took 5 days for the bacterium to become evident on XIM. Cultural characteristics of both isolated bacteria are shown in Table 1.

Plate 3. Cultural characteristics of the bacteria isolated from *Duranta repens*

Table 1. The cultural characteristics on various media of the bacterium isolated from *Duranta repens*

BASES	MEDIA										
	YDCA		SPA		NGA		KMBA		XIM		NA
	Yellow	White	Yellow	White	Yellow	White	Yellow	White	Yellow	White	
Amount of growth	Abundant	Abundant	Abundant	Scantly	Moderate	Moderate	Abundant	Abundant	Scantly	Scantly	Abundant
Form	Circular	Circular	Irregular	Circular	Circular	Circular	Circular	Smooth	Circular	Circular	Circular
Elevation	Raised	Raised	Convex	Convex	Convex	Convex	Convex	Raised	Raised	Raised	Flat
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Smooth	Smooth	Raised	Entire	Smooth	Smooth	Smooth	Entire	Entire	Entire	Smooth
Color	Light yellow	White	Cram	Cream	Yellow	Yellow	Dark yellow	Yellow	Light cream	White	Yellow/ whitish
Colony diameter (mm)	-no-*	-no-	72	30	34	44	67	24	22	20	25

*no = not observed

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Gram Stain Reaction

Both bacteria were Gram negative short rods with rounded ends. According to Agrios (1992) bacterium that cause leaf spots or blight were often Gram negative and may belong to either *Pseudomonas* or *Xanthomonas*.

Gram stain is one of the most important staining techniques in microbiology (Pelczar *et al*., 1983). It is a useful staining procedure in bacteriology and very essential for initial identification of the unknown bacteria (Raymond *et al*., 1991). Furthermore, Gram reaction of a bacterium is a good indicator of whether the bacteria can be destroyed using a given antibiotic.

The Gram negative cell wall has a more complicated structure than that of the Gram positive cell wall. It is composed of peptidoglycan, cytoplasmic membrane, periplasmic space and the outer membrane. Such structural component has a significant role in the pathogenicity, survival and antibiotic resistance of the Gram negative bacterium. Generally, many antibiotics will kill Gram positive bacteria, but Gram negative bacteria were often tougher to kill, resisting common antibiotics .

KOH Test

The preliminary classification of a bacterium as Gram-positive or Gram-negative is an essential step in both diagnostic microbiology and clinical medicine. The Gram stain is generally the first procedure performed in the identification of a bacterium and the results of this differential stain often determined the subsequent identification procedures. Often Gram stain yields equivocal results particularly with Gram-positive anaerobic bacteria and anaerobic Gram-negative bacteria. The major pitfall in the Gram stain is the tendency of some Gram-positive bacteria to decolorize more readily and be perceived

incorrectly as Gram negative. Some factors, e.g. composition of the growth medium and age of the culture (Conn *et al*., 1957) can influence the tendency of Gram-positive bacteria to decolorize.

Another rapid method for the preliminary classification of bacteria is the use of a 3 % solution of potassium hydroxide (KOH) (Gregerson, 1978). Like the Gram stain reaction, the KOH test is based on the differences in the chemistry of the bacterial cell wall. The cell wall of Gram-negative bacteria were easily disrupted when exposed to dilute alkali solutions resulting in the release of viscous “stringy” DNA.

Results of the KOH test show that walls of the yellow isolate were disrupted by the KOH, thus validating the Gram stain results. The white isolate was negative.

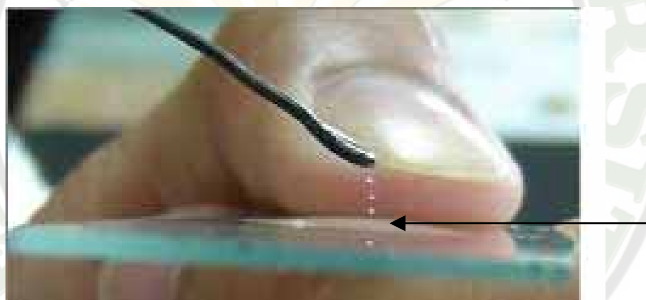


Plate 4. The positive KOH test on the yellow isolate

<u>Motility Test</u>

Both isolated bacteria were motile. The bacterium has a single polar flagellum. This is a diagnostic trait of *Xanthomonas* (Holt, et.al., 1994). The flagellated bacteria can move from one site to another on thin water films on plant or soil surfaces. Some bacteria can move through liquid media by means of flagella, whereas other have no flagella and cannot move themselves (Agrios, 1998).

Physiological Characteristics

The isolated bacteria were grown on various standard medium to determine its physiological characteristics. Since enzymes produce all physiological (biochemical) reactions in organisms and since individual genes produce each enzyme, we are essentially formulating a genetic profile of an organism (Benson, 1998), as we discover what enzymes are produced. Most results were determined by color changes effected by growth of the bacterium in various media (Table 1).

Gelatin Liquefaction Test. The organisms were not able to liquefy the gelatin even after 21 days of incubation. Therefore, the enzyme *gelatinase* was not produced (Plate 5).

Plate 5. Inoculated gelatin tubers (3-7, 14-21 days of incubation)

Starch Hydrolysis. The bacteria were streaked on starch agar. After 72 hours of incubation. Gram's iodine was poured over the growth on the medium. The presence of zones of clearing was observed around the bacterial growth (Plate 7).

Iodine solution (Gram's) is an indicator of starch. When iodine comes in contact with a medium containing starch, it turns blue. Starch is hydrolyzed and is no longer

present however, if the medium will have a clear zone next to the growth. Bacteria that hydrolyze starch produce amylases that yields maltose, glucose and dextrins (Benson, 1995).

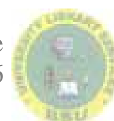
The ability to produced amylase is not uniformly present in all species of the genus *Xanthomonas*, but it is present in at least 75% of the strains from *X. campestris* complex (Swings, et.al., 1993).

Plate 6. Growth of the isolates on starch agar (72 HAI). The yellow isolate is on the left [positive] while the white isolate is on the right [negative]

Anaerobic Growth Test

Hugh and Leifson proposed this medium (Merck, 2003) for detecting oxidative and fermentative carbohydrate degradation. It is used primarily for the differentiation and classification of Gram-negative bacteria. A yellow coloration in both, the open and paraffin-sealed tubes, signifies fermentative degradation whereas yellow coloration of the open tubes alone indicates that the carbohydrate in question was broken down by oxidation. Oxidative breakdown took place at or close to the surface of the medium, whilst fermentative breakdown occurs both at the surface and throughout the butt.

Yellow isolate changes in color for both covered and unsealed, while the white isolate remains blue (Plate 7). These results were not conclusive and therefore, should be



repeated in future studies. The variable results may be attributed to error in the set-up and preparation of the test.

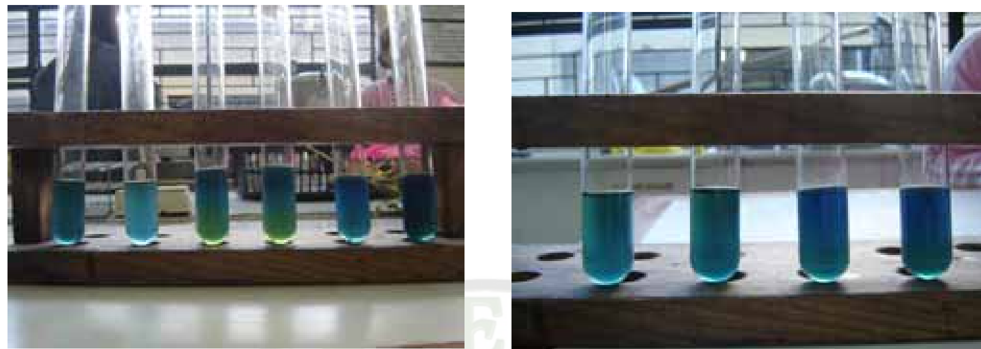


Plate 7. The comparison between yellow isolate (positive) and white isolate (negative)

Pathogenicity Test Result

Inoculation of either bacterium did not result in symptoms even using various means of inoculation, however, dipping of leaves in a combined bacterial suspension of the two isolates resulted in symptoms after 48 hours of incubation.

Table 2. Results of the various inoculation method

Inoculation Trials	Methods of Inoculation	Inoculum	Occurrence of Symptoms
1	Rubbing on leaves	Yellow	-
		White	-
2	Injecting on stem and leaves	Yellow	-
		White	-
3	Dipping of roots	Yellow	-
		White	-
4	Dipping of leaves	Both white and yellow isolates	+

- no symptoms
+ symptoms shown

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

One yellow isolate and one white isolate were obtained from symptomatic *Duranta repens* and grown on various media. The bacteria differed in cultural and physiological characteristics.

Conclusion

Based on the different tests done, the yellow isolate has characters typical of a *Xanthomonas campestris* pathovar while the white isolate most closely resembles *Pseudomonas sp*.

Since both bacteria did not produce the typical symptoms when inoculated separately and both were isolated at the same time from the symptomatic *Duranta*, it can be assumed that we may be dealing with a complex.

Recommendations

We have gained better perspective of the bacteria associated with bacterial angular spot or blight. Limitations of the study requires further study on the following:

1. Use of the combined inoculum from both bacteria and verification of the methods of inoculation.
2. Use of DNA sequence identification for specific taxonomic purposes.
3. The use of computerized identification (Duncan & Torrance, 1992) is suggested. Some strains of pathogen have variable reaction with almost all substrate so the use of dichotomous keys based on negative or positive results for individual test often leads to false identification.

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APPENDICES

A. YEAST EXTRACT DEXTROSE CACO3 AGAR (YDCA)

Components	g/l
Peptone	2.0 g
Dextrose (glucose)	20.0 g
Calcium carbonate, USP light powder	20.0 g
Agar	15 g
Sterile distilled water	1000 ml- 500 ml

B. KING'S MEDUIM B AGAR (KMBA)

Components	g/l
Protease peptone	20.0 g
K_2HPO_4	1.5 G
$MgSO_47H_2O$	1.5 g
Agar	15.0 g

C. NUTRIENT GLUCOSE AGAR (NGA)

Components	g/l
Agar	20.0 g
Glucose	2.5 g

D. SUCROSE PEPTONE AGAR (SPA)

Components	g/l
Sucrose	20.0 g
Peptone	5 g
K_2HPO_4	0.5 g
$MgSO_47H_2O$	0.25 g
Agar	15 g

Adjust to pH 7.2

E. XANTHOMONAS ISOLATION MEDIUM (XIM)

Components	g/l
Cellobiose	10.0 g
NaH_2PO_4	0.5 g

$MgSO_4 7H_2O$	0.3 g
K_2HPO_4	3.0 g
NH_4Cl	1.0 g
Agar	15.0 g

F. STARCH AGAR

Components	g/l
Soluble starch	10.0 g
$NH_4H_2PO_4$	0.5 g
NaCl	5.0 g
Water	1 liter
Yeast Extract	5.0 g
$MgSO_4.7H_20$	0.2 g
Agar	15 g

pH to 6.8

G. HUGH AND LEIFSON AGAR (ANAEROBIC GROWTH)

Components	g/l
Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Agar	3.0

Bromthymol blue (1% aqueous solution) 3 ml

pH 7.1