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Effect of X-ray Irradiation on Populations of *Pseudomonas amygdali* pv. *loropetali* pv. nov.

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Summary

Loropetalum, *Loropetalum chinense* (R. Br.) Oliv., is a popular landscape plant, but it can be infected by the gram-negative bacteria *Pseudomonas amygdali* pv *loropetali* pv. nov. Bacterial diseases are difficult to control, and this particular bacterium usually leads to disposal of the plant, resulting in economic losses for the nursery. These bacteria are causing galls on loropetalum which can cause stem girdling leading to reduced growth and possibly death of the plant. The bacteria will infect the plant if it can permeate through a cut or wound in the bark. This creates a major avenue for disease transmission when propagating from cuttings if cuttings are taken from infected plants. It is important that nurseries use proper sanitation steps to reduce the number of infested plants. One of the best ways to begin those sanitation steps is to start with clean cutting material. With growing public concerns on chemical pesticides and their residues, irradiation is becoming a viable alternative and an effective nonchemical treatment for the control of several path-

ogens. Studies have shown successful results when gamma irradiation was applied to *Pseudomonas* spp., therefore we hypothesize that radiation could eliminate *P. amygdali* pv *loropetali* pv. nov. on loropetalum stock plants. Bacteria were subjected to six levels of x-ray irradiation 0, 0.5, 1, 1.5, 2, 2.5 kGy (0, 500, 1000, 1500, 2000, 2500 Gy). Initial results showed that x-ray treatment to pure bacteria strains resulted in significant bacterial reduction at all levels, with complete inactivity being observed in the 1.5, 2, and 2.5 kGy (1500, 2000, and 2500 Gy) treatments. With these preliminary findings, further studies are being conducted to determine the application of radiation's ability to clean up infected loropetalum plant material.

INTRODUCTION

Loropetalum, *Loropetalum chinense* (R. Br.) Oliv., is a popular landscape plant for USDA Hardiness zones 7 – 10 (Dirr, 2017). The plant is native to eastern Asia and can range in height from dwarf-trailing varieties to upwards of 3.1 m (10 ft). Flower colors can range from white to various shades of pink, purple, or red. The petals are "fringe-like" hence, it is commonly referred to as Chinese fringe flower. In the spring of 2012, in South Alabama, bacterial gall was observed on loropetalum with symptoms similar to observations made on loropetalum in central Alabama, North Carolina and Georgia in nursery and landscape plants (Conner et al., 2013).

Nursery growers reported dieback and plant death and upon further inspection, the plants with dieback had "galling and irregular dark callus formation on the lower stem and lower branches" (Conner et al., 2013). Bacterial colonies were isolated and after testing measures, the bacteria *Pseudomonas savastanoi* was identified as the cause of the galls; this was the first report of *P. savastanoi* causing bacterial gall on loropetalum (Conner et al., 2013). This bacterium will form galls which can cause girdling of the stem leading to reduced growth and eventually death of the plant. In 2018, it was determined, based on pathogenicity assays and molecular tests, that the gram-negative bacteria *P. amygdali* pv *loropetali* pv. nov., are causing the gall on loropetalum (Harmon et al., 2018).

Pseudomonas generally like moist, warm environments (Ramos et al., 2012). The bacteria can be spread by rain or overhead irrigation splashing on to other plants. The bacteria will infect the plant if it can penetrate through a cut or wound in the bark, from there it forms a gall. It is important that nurseries take proper sanitation steps to reduce the number of infested plants. Currently, the best sanitation method is to throw away plants that are contaminated and completely remove them from the nursery. If these plants remain and cuttings are taken from them, it could further spread the bacteria. Also, the areas on the stock plant where the cuts were made are also now open to re-infection. Pruners and other cutting tools should be cleaned and disinfected to prevent the spread of the bacteria from plant to plant. Improper sanitation of

these instruments will only amplify the problem. Copes et al., (2019) observed that Clorox at 11% and Virkon S at 1.0% were able to completely eliminate *P. amygdali* pv *loropetali* pv. nov. on stainless steel, and Green-Shield II at 0.5% and KleenGrow at 0.8% nearly eliminated the bacterium on stainless steel. Based on this research, we know that there are "several disinfectants commercially available that can kill *P. amygdali* pv *loropetali* pv. nov. on production surfaces (Copes et al., 2019).

Pickens et al., (2019) reported that in 2013, several large nursery growers disposed over $1,000,000 worth of infected plant material. This disposal was either done by regulatory enforcement or was voluntary (Pickens et al., 2019). Bacterial diseases are difficult to control and currently there are no published recommendations for controlling this disease (Pickens et al., 2019). Auburn University recommends the use of copper treatments in the spring especially after pruning or other events causing damage to the bark (Pickens et al., 2019).

With growing public concerns on environmental and health issues stemming from chemical pesticides and their residues, and also the development of bacterial resistance, irradiation has become a viable alternative and an effective nonchemical treatment for the control of several pathogens (Hallman, 2011; Jeong et al., 2016).

Irradiation causes cellular damage, with direct effects and with indirectly generating reactive oxygen species, which would cease their metabolic functions (Jeong et al., 2016). The unit of irradiation dose is the gray (Gy), which is the energy absorbed in joule of radiation energy per kilogram of matter (Jeong et al., (2016). Gamma irradiation has been successfully observed to inhibit the growth of fungal and bacterial pathogens on fruits or vegetables, such as *Botrytis cinerea* in sweet pepper, *Penicillium purpurogenum* in pineapple, *Rhizopus stolonifer* in sweet potato, *Pseudomonas syringae* pv. *tomato* (*Pst*). in tomato, *Pseudomonas fluorescens* in baby spinach and romaine lettuce and *Monilinia fructicola* in peach (Damayanti et al., 1992; Jeong et al., 2015; Jeong et al., 2016; Kim and Yook, 2009; Olanya et al., 2015; Yoon et al., 2014). Applying gamma irradiation at 25 kGy (25000 Gy) is a common procedure for the sterilization of many food products and medical applications (Jones et al., 2010). McNamara et al., (2003) inferred that most bacteria are eliminated at levels from 15 to 25 kGy (15000 to 25000 Gy). Jeong et al., (2016) observed that gamma irradiation levels of 0.15 kGy (150 Gy) successfully controlled *P. syringae* pv. *tomato* (*Pst*). in tomato seedlings with no phytotoxicity observed. *Pseudomonas fluorescens* was controlled when levels were 0.04 to 0.05 kGy (40 to 50 Gy) and 0.05 to 0.06 kGy (50 to 60 Gy) in baby spinach and romaine lettuce respectively (Olanya et al., 2015). The same species of *Pseudomonas* was also controlled in radiated beef steaks at levels of 1.5 and 3.0 kGy (1,500 and 3,000 Gy) (Chung et al., 2000).

Based on the results of gamma irradiation applied to *Pseudomonas* in these studies, we hypothesize that radiation could eliminate *P. amygdali* pv *loropetali* pv. nov. on loropetalum stock plants. Successfully eliminating plant pathogens using radiation could save nurseries substantial money.

MATERIALS AND METHODS

Standard operating procedures for a sanitary microbiology laboratory were followed in order to encourage aseptic processes

(Plant Bacteriology Specialist, 2022). Suppression of bacterial colony formation could be observed if levels of contamination are high. Pure colonies of *Pseudomonas amygdali* pv *loropetali* pv. nov. were obtained from Dr. Kassie Conner, Auburn University, in March and June 2022. Dr. Conner verified the strain of bacteria using PCR. Colonies were maintained on Pseudomonas F (BD Diagnostic Systems) nutrient agar filled petri dishes. Liquid culture, utilizing Tryptic Soy Broth (TSB), were inoculated with colonies of *P. amygdali pv loropetali pv. nov.* and placed in a 25°C incubator 72 hours prior to irradiation.

The day of irradiation, the concentration of bacteria in the liquid culture was determined using a spectrophotometer (Evolution 60S UV-Visible Spectrophotometer). TSB was used as the blank in the spectrophotometer and the bacteria suspended in TSB were adjusted to an optical density of 0.302 at 420nm (OD_{420}). Based on calculations by Dr. Shien Lu, Mississippi State University, this would result in approximately $2x10^8$ cells per ml (Plant Bacteriology Specialist, 2022). Next, 10mL of the liquid culture were placed into petri dishes for irradiation in the Kimtron 350 X-ray (Kimtron Inc, Oxford, CT, USA). The Kimtron 350 utilized is a customized X-ray irradiator for food irradiation research (Wu and Chang, 2020).

Petri dishes were exposed to 6 levels of irradiation: 0, 0.5, 1, 1.5, 2, or 2.5 kGy (0, 500, 1000, 1500, 2000, or 2500 Gy). Each treatment contained three petri dishes of bacteria, with a total of 18 dishes irradiated (**Fig. 1**). Along with the three petri dishes, three alanine markers (dosimeters) were also placed with the samples to record the amount of radiation to which the samples were exposed (**Fig. 2**).

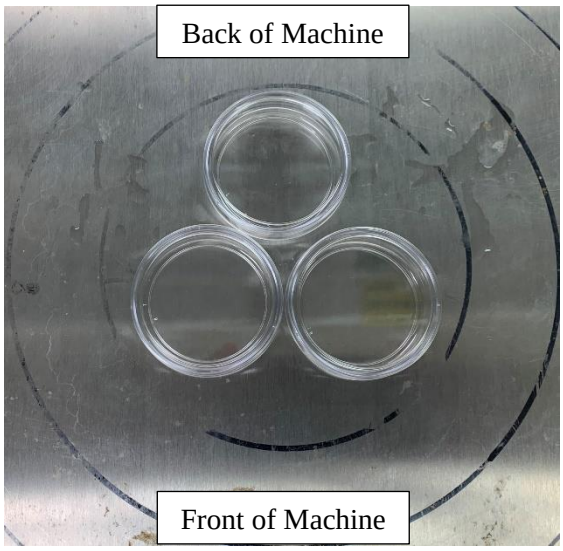

Figure 1. Plate orientation in Kimtron IC 350 X-ray machine.

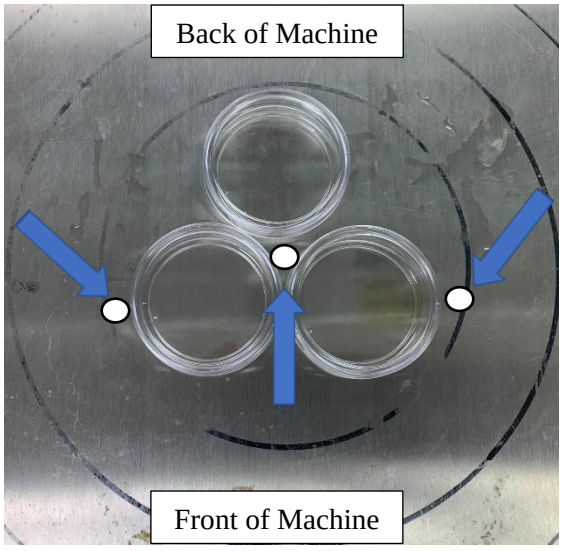

Figure 2. Representation of placement of dosimeters in Kimtron IC 350 X-ray machine.

After irradiation treatements, bacteria were transferred to 15mL tubes and placed in a 2°C (37°F) refrigerator for 24 hours. Tubes were then removed and six serial dilutions and six platings were done for each tube containing the irradiated liquid culture. Plates were then placed back in the

incubator for cultures to grow for 72 hours at 25°C. Plates were removed from the incubator and colony forming units (CFUs) were counted using a Reichert Darkfield Quebec Colony Counter (RE-3325). Data were analyzed using SAS 9.4 PROC GLIMMIX and pairwise comparisons were done using Tukey's HSD where our alpha was 0.05.

RESULTS AND DISCUSSION

The levels chosen for this experiment were based on previous research. *Pseudomonas fluorescens* was controlled when levels were 0.04 to 0.05 kGy (40 to 50 Gy) and 0.05 to 0.06 kGy (50 to 60 Gy) in baby spinach and romaine lettuce respectively (Olanya et al., 2015). *Pseudomonas fluorescens* was also controlled in radiated beef steaks at levels of 1.5 and 3.0 kGy (1500 and 3000 Gy) (Chung et al., 2000). It was also reported to be controlled at irradiation levels of 1.2 - 2.3 kGy (1200 – 2300 Gy) (Frazier and Westhoff, 1988). In our experiment, we observed significant control of *P. amygdali* pv *loropetali* pv. nov. in all treatments compared to our non-irradiated control (**Table 1**; $P<0.0001$).

Our preliminary results are similar to reports by Jeong et al., (2016) who observed that CO_{60} gamma irradiation levels of 0.1 – 0.2 kGy (100 - 200 Gy) successfully controlled *P. syringae* pv. *tomato* (*Pst*). in tomato seedlings with no phytotoxicity observed. They observed that their treatment of 0.1 kGy (100 Gy) resulted in an approximate 5-log reduction of the viable count compared to the initial counts and complete inactivity at 0.2 kGy (200 Gy), which was their lethal dose (Jeong et al., 2016).

Using X-ray irradiation, we observed a 2.38-log reduction from our control colony counts compared to the counts of our first treatment of 0.5 kGy (500 Gy). At 1.5 kGy (1500 Gy) all bacterial colonies were rendered completely inactive. The differences in levels needed to inactivate our respective bacteria could be due to we were each experimenting with a different *Pseudomonas* sp. and sources of radiation, while similar, utilized different radiation sources.

Table 1. X-ray irradiation effect on average log of colony forming units.

Irradiation Level (kGy)	Mean Log CFU/mL[z]
0 (Non-irradiated Control)	3.02 a
0.5	0.64 b
1.0	0.41 b
1.5	0.0 b
2.0	0.0 b
2.5	0.0 b
P-value[y]	<0.0001

[z]Analysis of variance was performed using PROC GLIMMIX (SAS 9.4). Means followed by the same letter are similar and not significantly different ($\alpha = 0.05$). Each irradiation level had three replications. [y]P values for differences between means were obtained using Tukey's honest significant difference (HSD) at $P \leq 0.05$.

According to the EPA (2022), X-rays and gamma rays have the same basic properties, however, they are generated from different parts of the atom with X-rays being emitted from processes outside the nucleus, and gamma rays originating inside the nucleus. X-rays also are generally lower in energy and can be less penetrating than gamma rays, explaining why our required doses to reach bacterial inactivity were so

much higher than Jeong et al., (2016) observed (EPA, 2022).

Knowing that we can irradiate and effectively control *P. amygdali* pv *loropetali* pv. nov. provides a foundation for future work examining radiation as a source of plant pathogen control. Further bacterial biological replications are currently being irradiated, as well as loropetalum cuttings, to provide us with further confirmation on optimal irradiation levels. If treatment with irradiation can provide growers with clean stock plants for cuttings, it is possible this treatment could provide a viable control option for loropetalum gall to limit economic losses for nurseries.

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