IDENTIFICATION AND MANAGEMENT OF CROWN GALL DISEASE (*Rhizobium* sp.) OF KIWIFRUIT (*Actinidia deliciosa* LIANG AND FERGUSON) IN DOLAKHA, NEPAL

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ABSTRACT

The crown gall disease of kiwifruit, caused by the bacterium *Rhizobium* (formerly *Agrobacterium*), is a significant plant disease that affects the roots and stems, leading to tumor-like growths. In June 2022, a survey of kiwifruit nurseries was conducted in Boch, Bhimeshwar, Dolakha. The survey covered 20 nurseries from three farms to observe the incidence of crown gall disease in the area. Subsequently, samples were collected and analyzed at the laboratory. The findings reveal that the isolated bacteria produced distinct creamy white colonies on PDA and exhibited a positive KOH test and Gram-negative staining. Pathogenicity tests confirmed the presence of crown gall symptoms in carrot disks and tomato seedlings. Hence, the bacterium was found to be crown gall disease of kiwi caused by *Rhizobium* sp. Then, field research was conducted in a two-factorial randomized complete block design with three replications at the Temperate Fruits Rootstock Development Centre at Boch, Dolakha to find out the effective control measures for crown gall disease (*Rhizobium* sp.) management in Kiwifruit nursery. The first factor included six treatments (Kasu B, Hydrogen peroxide, Cuprex, Streptocycline, Kasu B + Streptocycline and untreated control). The second factor included three varieties of Kiwifruit (Hayward, Monty and Allison). In the field experiment, observations were taken on plant height, root length, colony count before and after the treatments, number of galls, and size of galls. The interaction of varieties and treatments on the disease management was significant ($p<0.05$). The highest bacterial population reduction (100%) was found in the plots of Allison treated with Streptocycline + Kasu B followed by Streptocycline-treated plots of Hayward. The Streptocycline showed the lowest gall numbers (2.67 ± 0.69) in Allison followed by Kasu B (6.76 ± 0.78). The Cuprex-treated Allison variety showed the smallest gall size (0.23 ± 0.40 cm) which was at par with Cuprex-treated Hayward (0.29 ± 0.50 cm) and Monty (0.32 ± 0.56 cm) varieties. In overall, Allison variety was found comparatively less susceptible to crown gall disease and Streptocycline was found to be effective in mitigating crown gall disease in Kiwifruit compared to other treatments.

Key words : *Rhizobium,* crown gall, Kasu B, kiwi, streptocycline

INTRODUCTION

Kiwifruit (*Actinidia* spp.) originating from China is a deciduous vine that was introduced to the world market from New Zealand in the 1950s (Barboni, 2010). In Nepal, kiwifruit is found to be successfully cultivated at an altitude of 1200 to 2500 masl. It has gained popularity among Nepalese farmers in recent years and the kiwifruit planting area has increased significantly (Sharma et al., 2020). A recent data shows kiwifruit has been cultivated in 3291 ha with 22015 mt production and 6.69 mt/ha productivity in Nepal (MoALD, 2023). Though introduced in 1986 AD, commercial kiwifruit cultivation started in Nepal only in 2009 AD (Atreya et al., 2020).

Crown gall, a disease caused by the bacterium *Rhizobium* (formerly *Agrobacterium*) was found to be rapidly spreading in nursery plants in Dolakha. It was identified at the laboratory of Nepal Plant Disease and Agro Associates (NPDA), Balaju-Chakrapath, Kathmandu in 2020 (Manandhar, 2020). This pathogen forms galls on the root and crown parts of plants which upon expanding, impair the vascular system of the plant. Thus, the absorption and transportation of water and minerals become affected in the diseased plants, resulting in weakening and ultimately death of plants (Kahla et al., 2017). Also, diseased plants are more sensitive to secondary infection and environmental stress (Carroll and Wong, 2018). The present study was conducted with an objective of identifying the bacteria and evaluating some chemical options under field conditions.

MATERIALS AND METHODS

Survey and Collection of Crown Gall and Soil Samples

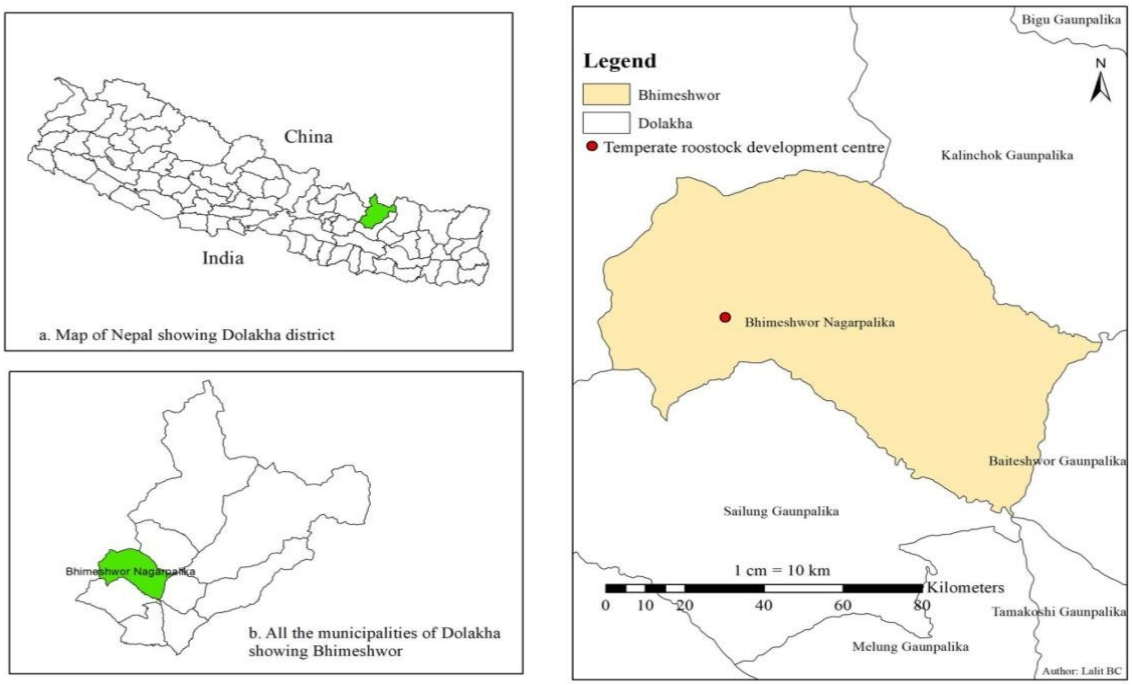


Fig. 1. GIS map of research field

A general survey was conducted in 11 nurseries in two private farms, and 9 nurseries of the Temperate Fruits Rootstock Development Center (a Government farm) at Bonch, Dolakha in Bhimeshwar-08, Dolakha in June 2022 (Fig. 1). Ten plants from each plot were examined for the presence of crown gall symptoms. The crown gall and the soil samples around the root zone of the plants with galls from each nurseries were collected. The samples were brought to the laboratory of Nepal Plant Disease and Agro Associates (NPDA) and stored at 5°C until processing.

Isolation of Crown Gall Pathogen from Crown Galls and Soil

The samples were thoroughly washed with tap water to remove intact soil, surface sterilized with freshly prepared 1% sodium hypochlorite followed by rinsing with sterilized water, and left for 10-15 minutes for drying under laminar air flow. Then, a sterilized petri dish with a few drops of sterilized water was taken and the gall was crushed into it using a sterilized needle. A loopful suspension was streaked on potato dextrose agar (PDA) plates.

For the isolation of the bacteria from soil samples, 1 g of soil from each sample was mixed in a test tube containing 9 ml sterilized water and serially diluted up to 10^{-3} concentrations. The serially diluted suspension (100 µl/plate) was plated onto D-1 agar plates (Kado and Heskett 1970), and incubated at 28 °C for 48 hours. Typical colonies resembling *Rhizobium* sp. (Kado and Heskett 1970; Chandran and Sharma, 2015) were counted and cultured on PDA. In total, four isolates were maintained in PDA. The bacterial cultures were subjected to Gram's staining (Smith and Hussey, 2020) and KOH (Arthi et al., 2003) tests for confirming the Gram reaction.

Pathogenicity Tests

The tests were done in tomato plants and on carrot discs. The tomato variety, Manakamana, was grown in pots. At the three to four-leaf stage of 20-day seedlings, a wound was made on the crown part using a sterilized needle and the suspension of 24-hour-old bacteria (10^9 cfu/ml) was inoculated into the wounded part. The inoculated plants were kept under natural environment to observe development of symptoms. Un-inoculated plants were maintained as control.

For carrot assay, carrot was brought from a local market, peeled, washed, and flamed briefly with 95% ethanol. Then, the surface was sterilized in freshly prepared 1:10 dilution of household bleach (containing 4% w/v sodium hypochlorite) for 15 minutes followed by washing with three changes of sterile water. The carrot root was cut into 5 mm thick slices perpendicular to the axis of the root and placed on moistened sterile filter paper in Petri dishes. The cut portion was inoculated with the freshly prepared bacterial suspension (10^9 cfu/ml) and placed in an incubator at 26-28 ^oC. Observation was made for tumor formation around the inoculated sites after 3 weeks.

In-vitro Chemical Sensitivity Test of the Bacteria

Sensitivity of the bacteria to some chemicals was performed by the Kirby-Bauer diffusion susceptibility test. The test was done in Mueller Hinton (MH) agar (Hudzicki, 2009). One-day-old bacterial suspension was prepared in saline water (0.9%) in a tube. A sterile cotton earbud was dipped into the suspension tube. The earbud was rotated against the wall of the tube (above the fluid level) by gentle pressure to remove excess bacterial suspension. Inoculation was done on the dried surface of the MH agar plate by streaking the cotton earbud over the entire surface.

After the inoculation, filter paper disks of 5 mm diameter were dipped in the treatments (test chemicals) with different concentrations (10,000 ppm, 1000 ppm, 500 ppm, 200 ppm and 100 ppm). The chemicals used were Kasu B (kasugamycin), Hydrogen peroxide, Cuprex (copper oxychloride), Streptocycline (streptomycin + tetracycline), and Kasu B + Streptocycline. The disks were gently placed on the inoculated MH agar plate using sterilized forceps. This test was conducted in a completely randomized design with five replications (one plate as one replication).

Efficacy Test of Chemicals against Crown Gall in Nurseries

An experiment was conducted to find out the effective control measures for crown gall disease of kiwifruit in nurseries at the Temperate Fruits Rootstock Development Center, Bonch, Dolakha.

The research was conducted on crown gall-infected kiwifruit nurseries. The saplings were grown in rows on raised beds where each row consisted of at least 20 saplings. Each row was considered an experimental plot for each treatment. The row-to-row and plant-to-plant spacing were 40 cm and 15 cm, respectively. The experiment was organized in a two-factorial randomized complete block design with three replications. The factors included three varieties - Hayward, Monty and Allison, and six chemical treatments (table 2), including untreated control.

The treatments were mixed with water at the specified dose and 20 liters of solution/suspension was applied thoroughly in the soil around the root zone in each plot. Treatments were applied four times during the experiment at one-month intervals. In the untreated control plots, water was applied.

Table 2. Treatment details used in the field experiment on management of crown gall in kiwifruit at Bhimeshwor, Dolakha, Nepal, 2022

Treatments	Application dose (Formulation)
Kasu B (kasugamycin 3% SL)	1 ml/L
Hydrogen peroxide 6.5% W/V	4.5 ml/L
Cuprex (copper oxychloride 50% WP)	1 g/L
Streptocycline (streptomycin 90%+tetracycline 10% SP)	0.5 g/L
Kasu B + Streptocycline	0.5 g/L + 0.25 g/L
Untreated control (water)	-

Soil Sample Collection for Bacterial Population Count

Each time, before and after the application of treatments, soil samples were taken to determine the bacterial population. About 100 g of soil was collected from around the plant root. Five sub-samples were collected from five random spots in each plot of the kiwifruit nursery making about 500 g soil samples from each plot (replication). The collected soil samples from each plot of the individual treatments were mixed and made a composite sample of 500 g for each treatment. Then, the samples were brought to the NPDA laboratory to count the bacterial population. The bacterial count was done D-1 agar plate as described earlier.

Assessment of Plant Growth and Crown Gall

Five plants were randomly selected and uprooted from each plot at the time of final data collection. Their plant height, root length, gall number per plant, and gall size were recorded. The diameter of each gall was measured from two sides and an average was taken.

Statistical Analysis

The data were tabulated in Microsoft Excel 2016. The data analysis was done on R programming under RStudio interface using 'doebioresearch' package to test the significance of treatments and to compare means. The graphical representation was done by using Microsoft Excel and Statistical Package for the Social Sciences (SPSS) version 25.

RESULTS AND DISCUSSION

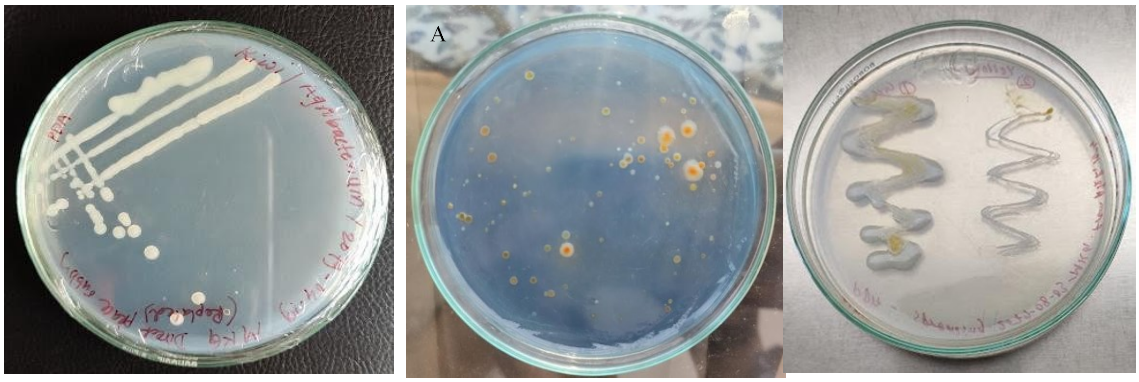
Survey of Crown Gall in Kiwifruit Nurseries

All 20 nurseries surveyed had crown gall symptoms. Of the total 200 saplings examined in 20 nurseries, 107 saplings (53%) had the symptoms. The gall diameter ranged from 2 to5 cm, which were rough, light to dark brown, and covered with dead tissue and the inner part contained a white, fleshy callus. The present results revealed that the crown gall disease was spread in the surveyed areas. Manandhar (2017) in his report also mentioned the occurrence of the disease in various parts of Dolakha district. This suggests the crucial need to produce crown gall-free healthy seedings/saplings and impose domestic quarantine to prevent the spread of the disease from infested areas to disease free areas.

Isolation and Identification of Crown Gall Pathogen

On isolation directly from the crown gall, creamy white colony was observed on PDA plate on the next day of streaking (Fig. 2a). On isolation from soil, circular, convex and shiny colonies surrounded by whitish ring (looked-like poached egg) grew on D-1 agar medium (Fig. 2b) was observed 2 days after spreading. At initial stage, the colonies were light blue which changed to greenish yellow color in 4-5 days. Also, some colonies were completely yellow and changed the color of medium from blue to yellow around the colonies. When a typical colony was streaked onto PDA plate, creamy white growth was seen (Fig. 2c).

Both the 3% KOH solubility test and Gram staining confirmed all the bacterial isolates were Gram positive. On inoculation test, the bacteria developed tumor-like growth around the inoculated site on carrot slices 3 weeks after incubation (Fig. 3a). Similar result was found by Limanska et al. (2015) on carrot disc by *Agrobacerium* (Now *Rhizobium*). On tomato plants, it developed small galls on the crown region after 40 days of inoculation (Fig. 3b).



(a) (b) (c)

Fig. 2. (a) Growth of *Rhizobium* isolate from kiwifruit root on PDA plate, (b) Isolation of *Rhizobium* sp. on D1 agar plate, and (c) Growth of *Rhizobium* sp. (isolated from D1 agar plate) onto PDA plate

(a) (b)

Fig. 3. (a) White galls formed in a ring-like structure on carrot disks inoculated with *Rhizobium* sp., (b) Formation of gall on crown region of tomato seedlings inoculated with *Rhizobium* sp.

Based on the colony characteristics on D-1 agar medium, shape and colour of stained bacterial cells and the symptoms they produced on carrot slices and tomato seedlings, the bacteria causing crown gall in kiwifruit was confirmed as *Rhizobium* sp. (formerly known as *Agrobacterium* sp.). We could not determine the species as our investigation lacks detailed identification process. In the present study the bacterial colonies were found circular, convex, and shiny central mass surrounded by a whitish ring looking like a poached egg, which were similar to the descriptions for the colony morphology of the genus *Agrobacterium* (now called *Rhizobium*) reported by Kado and Heskett (1970) and Chandran and Sharma (2015).

In the present study, saprophytic yellow colonies of bacteria were also detected. According to Kado and Heskett (1970), saprophytic bacteria change the color of bromothymol blue from blue to yellow around them by the production of certain acid. We also detected similar changes with the saprophytic bacteria.

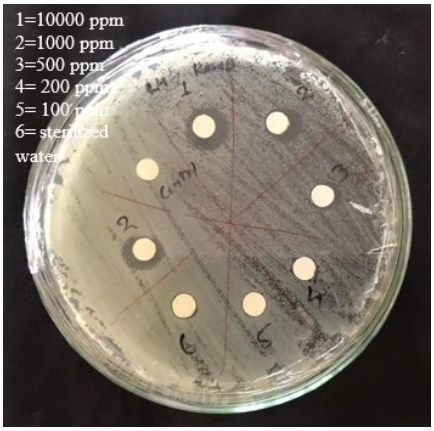
In Vitro Chemical Sensitivity Test of *Rhizobium* sp.

All tested chemicals significantly inhibited the growth of *Rhizobium* sp. (Table 3). Of them, Streptocycline at 10,000 ppm produced significantly highest inhibition zone. Also for other chemicals, their inhibitory effect increased with increased concentrations. The *in vitro* inhibition of bacterial growth by the tested chemicals at different concentrations is shown in Fig. 5.

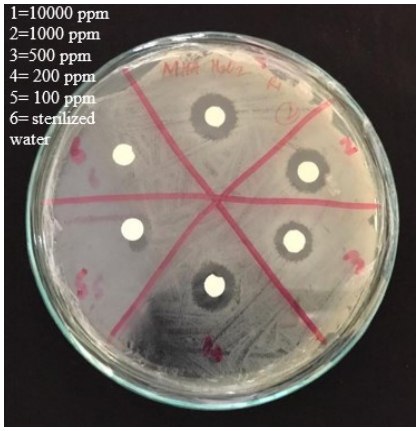
Table 3. *In vitro* inhibition of the growth (cm) of *Rhizobium* sp. by chemicals at different concentrations

Treatments	Average inhibition (cm) measured from the edge of disc to edge of inhibition zone at different concentrations (ppm)				
	10000	**1000**	**500**	**200**	**100**
Kasu B	1.03^{c} ±0.07	0.86^{b} ±0.05	0.69^{b} ±0.02	0.60^{b}±0	0.50^{c}
Hydrogen peroxide	1.73^{b}±0.12	1.52^{a}±0.14	1.07^{a} ±0.08	0.92^{a}±0.03	0.72^{a}±0.02
Cuprex	1.07^{c} ±0.05	0.92^{b} ±0.05	0.81^{bc}±0.05	0.76^{ab}±0.05	0.60^{b}±0.01
Streptocycline	2.33^{a} ±0.19	1.52^{a}±0.10	1.11^{a} ±0.13	0.94^{a}±0.13	0.76^{a}±0
Kasu B + Streptocycline	1.52^{b} ±0.05	1.30^{a} ±0.03	0.96^{ab}±0.07	0.82^{a}±0.05	0.61^{b}±0.02
Untreated control	0	0	0	0	0
F-test	***	***	**	*	***
LSD	0.33	0.25	0.24	0.20	0.04
CV%	16.34	15.67	19.37	18.95	5.20

Same letters in the treatments are not significantly different, ± = indicates standard error, CV= Coefficient of variation and LSD = least significant difference.



(a)



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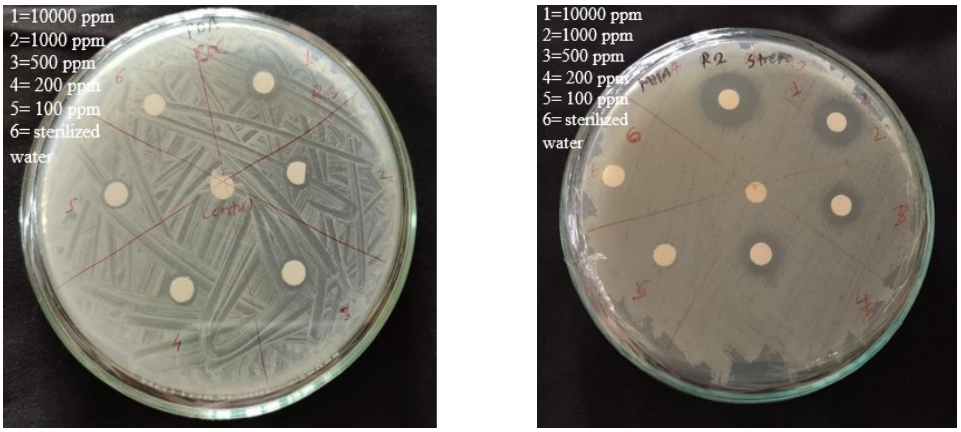

(c) (d)

Fig. 5. Inhibition of the growth of *Rhizobium* by (a) Kasu B (kasugamycin), (b) hydrogen peroxide, (c) Cuprex, and (d) Streptocycline (streptomycin + tetracycline)

Effect of Treatments and Varieties on Root Length, Plant Height, Gall Number and Gall Size

The interaction effect of varieties and treatments was not significantly different for root length and plant height (Table 4). The interaction was significantly different ($p<0.05$) for number and size of galls. The lowest average gall number was found on the saplings of cultivar Allison treated with Streptocycline (2.67) followed by Kasu B (6.76). The number of galls on Kasu B-treated saplings in Allison was at par with Cuprex-treated saplings (7.43). The untreated saplings of variety Monty had a maximum number of galls (30.80) which was followed by varieties Hayward (22.80) and Allison (20.47).

The smallest gall size was found in the Cuprex-treated Allison variety (0.23 cm) which was at par with Cuprex-treated Hayward (0.29 cm) and Monty (0.32 cm) varieties (Table 6). The largest gall size was found in untreated saplings of Monty (30.80 cm) which was at par with untreated saplings of Allison (1.36 cm). The gall size of untreated saplings of Allison was followed by untreated saplings of Hayward (1.14 cm).

Table 4. Interaction effect of kiwifruit varieties and treatments on different parameters, Bonch, Dolakha, Nepal, 2022

Treatments	Varieties	Root length (cm)	Plant height (cm)	Gall number	Gall diameter (cm)
Kasu B	Hayward	20.23(1.30)	42.44(1.60)	10.23^{ghij}±0.29	0.38^{ijk}±0.65
	Monty	21.63(1.34)	37.89(1.57)	12.10^{fgh}±1.16	0.73^{def}±1.27
	Allison	30.00(1.47)	49.67(1.69)	6.77^{k}±0.78	0.46^{hij}±0.78
Hydrogen peroxide	Hayward	20.80(1.30)	39.94(1.58)	12.10^{fgh}±2.06	0.68^{efg}±1.19
	Monty	20.53(1.30)	24.88(1.39)	14.80^{ef}±0.95	0.68^{efg}±1.17
	Allison	20.43(1.30)	35.89(1.55)	9.00^{hijk}±0.19	0.60^{fgh}±1.04

Treatments	Varieties	Root length (cm)	Plant height (cm)	Gall number	Gall diameter (cm)
Cuprex	Hayward	25.57(1.41)	43.44(1.64)	11^{ghi}±2.14	0.29^{jk}±0.50
	Monty	23.67(1.36)	28.89(1.46)	13.00^{efg}±1.50	0.32^{jk}±0.56
	Allison	21.00(1.32)	44.44(1.64)	7.43^{jk}±1.25	0.23^{k}±0.40
Streptocycline	Hayward	27.53(1.44)	41.11(1.60)	8.67^{ijk}±1.20	0.67^{efg}±1.15
	Monty	21.47(1.33)	30.22(1.48)	10.47^{ghij}±0.62	0.53^{ghi}±0.92
	Allison	26.13(1.41)	47.79(1.66)	2.67^{l}±0.69	0.56^{fghi}±0.96
Kasu B + Streptocycline	Hayward	19.00(1.26)	46.33(1.66)	18.90^{cd}±0.48	1.03^{bc}±1.78
	Monty	18.33(1.26)	25.44(1.40)	16.13^{de}±0.29	0.88^{cd}±1.52
	Allison	19.57(1.29)	30.89(1.49)	13.10^{efg}±0.62	0.79^{de}±1.37
Control	Hayward	20.67(1.30)	39.28(1.60)	22.80^{b}±0.68	1.14^{b}±1.98
	Monty	23.86(1.38)	35.00(1.54)	30.80^{a}±0.48	1.38^{a}±2.39
	Allison	17.10(1.23)	37.44(1.57)	20.47^{bc}±1.22	1.36^{a}±2.35
F-test		ns	ns	*	**
LSD				3.15	0.18
CV%				14.24	15.45
SEM				2.42	0.06

Same letters in the treatments are not significantly different, value after ± indicates standard error, CV is coefficient of variation and LSD denotes least significant difference. Value in the parenthesis indicates log transformed value.

Population Counts of *Rhizobium* in Soil before and after Treatments

Initially, the average bacterial population was more than 15 cfu per petri dish from each soil sample. After the three applications of the treatments within four months, the bacterial population decreased in all treatments (Fig. 6).

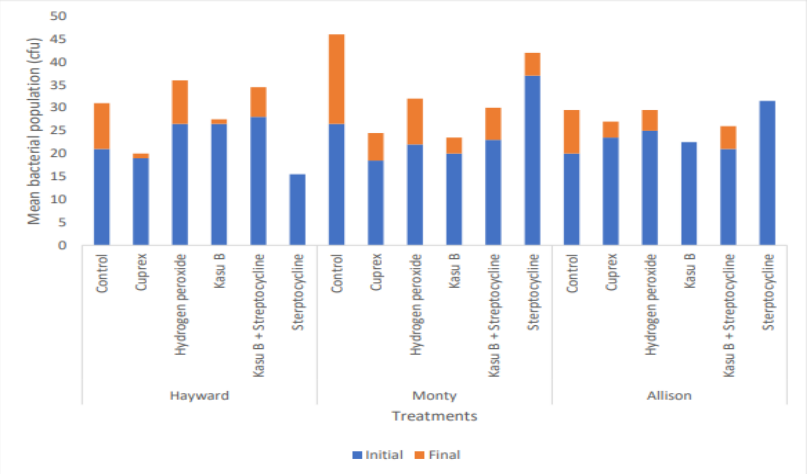

Fig. 6. Initial (before the start of treatment) and final (after four applications of treatments) counts of *Rhizobium* from soils

The highest bacterial population reduction was found in the soils of Streptocycline-treated plots of Hayward and Streptocycline + Kasu B-treated plots of Allison. Cuprex and Hydrogen peroxide showed similar reduction percent in Allison whereas the Cuprex reduced more bacterial population than Hydrogen peroxide in Hayward and Monty. The combination of Kasu B and Streptocycline showed a better reduction in Hayward and Monty than Hydrogen peroxide, however, it reduced less in Allison. The lowest reduction percent was found in untreated Monty followed by untreated Allison and Hayward. The better effect of treatments on variety Allison against the pathogen might be due to its ability to resist the pathogen more than the other two varieties (Fig. 7).

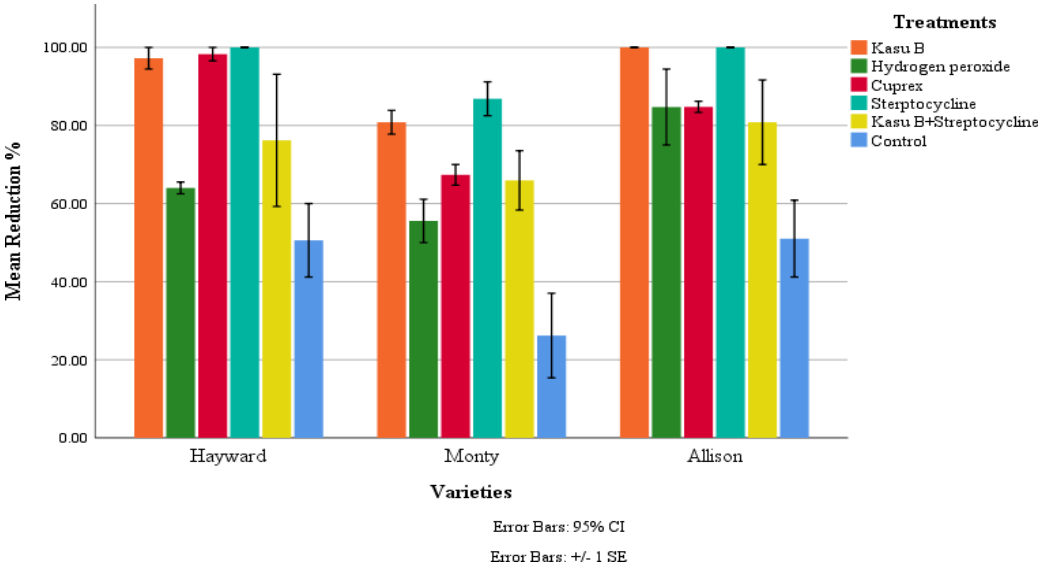

Fig. 7. Reduction percent in final counts of *Rhizobium* after four applications of treatments

The effects of the chemicals on decreased colony counts could be directly attributed to their antibacterial activity. It is also true that the bacterial counts were lower in the control plots (without treatment), but significantly higher than in treated plots, which could be attributed to soil moisture that affect bacterial growth and activity, was less at the time of final counts. Krimi et al. (2002) reported that bacterial population remain low or absent in the fall and winter seasons.

Kasugamycin causing the bacterial cell to die by attaching to the bacterial ribosome and blocking the protein synthesis (Schuwirth et al., 2006). Wang et al. (2021) also reported the antibacterial effect of kasugamycin in kiwifruit for different bacterial diseases including crown gall disease. The bactericidal effect of kasugamycin (Kasu B) was also reported by Adaskaveg et al. (2011) against a bacterium *Erwinia amylovora*.

The in vitro inhibition of bacterial population by hydrogen peroxide might be due to its strong oxidizing property. The bactericidal effect of hydrogen peroxide was also found by Rios-Castillo et al. (2017) against Escherichia coli, Staphylococcus aureus, Enterococcus hirae, and Pseudomonas aeruginosa. The potential of Hydrogen peroxide for bacterial control was also reported by Bosmans et al. (2016) against rhizogenic bacteria like *Agrobacterium* (Now called *Rhizobium*). However, the Hydrogen peroxide was found less effective under field conditions which be due to its volatile nature.

The antibacterial effect of Cuprex might be due to the release of reactive oxygen species by the copper ions that causes damage to cellular components of the bacteria. Also, it might be due to interference induced by the copper ions on enzymatic activity required for bacterial metabolism and growth (Arendsen et al., 2019). The antibacterial effect of Cuprex for controlling crown gall disease was also found by Utkhede and Smith (1993) in Apple.

The better performance of Streptocycline against the Rhizobium might be due to streptomycin and tetracycline present in it. The streptomycin works by binding to 16S rRNA of bacterial ribosome, impairing its functions and blocking further protein synthesis in bacteria (Ball et al., 1975; Germovsek et al., 2017; Luzzatto et al., 1968; Vianna et al., 2019). Similarly, tetracycline is also known to block the aminoacyl-tRNA from adhering to the bacterial ribosome, hence inhibiting the production of proteins by bacteria for their growth and multiplication (Chopra et al., 1992; Schnappinger and Hillen, 1996). Tanaka et al. (1995) reported the tetracycline as best antibiotic against *Rhizobium* (formely *Agrobacterium*) followed by other antibiotics, including streptocycline.

CONCLUSIONS

The crown gall disease is prevalent in kiwifruit grown in different parts of Dolakha district. The causal agent of the disease is identified as *Rhizobium* sp. (formerly *Agrobacterium* sp.) Chemicals tested varied for their efficacy in both *in vitro* and field tests. and Streptocycline was found the most effective. Among the three varieties, Allison was found relatively less susceptible to crown gall disease followed by Hayward and Monty. Imposing domestic quarantine is highly needed.

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