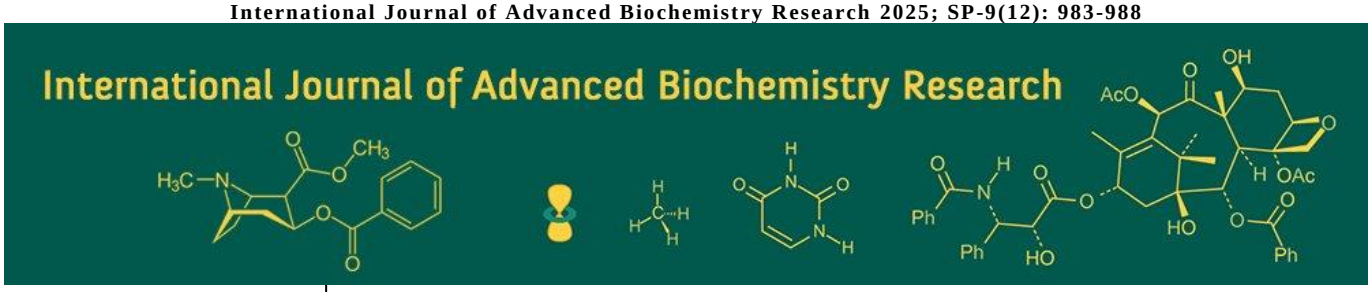

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In vitro management of *Pectobacterium carotovorum* subsp. *carotovorum* the causal organism of carrot soft rot disease using chemicals and antibiotics

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Abstract
The effectiveness of four chemical substances and five antibiotics was assessed *in vitro* against *Pectobacterium carotovorum* subsp. *carotovorum*, the pathogen responsible for carrot soft rot disease. Pectobacterium carotovorum subsp. carotovorum was isolated from diseased carrot samples using the serial dilution technique, and various biochemical and pathogenicity tests were conducted to confirm the bacterial species. chemicals *viz*., copper oxychloride, copper hydroxide, nano zinc and nano copper at 0.005, 0.01, 0.02 and 0.03 per cent and antibiotics *viz*., tagmycin, bacterinashak, plantamycin, kasugamycin, validamycin at 50, 100, 250, 300 and 500 ppm were tested against the pathogen by well diffusion method. Data were recorded at 96 hour after incubation and results showed that among the chemicals copper oxy chloride exhibited greater inhibition zone (9.68 mm) followed by nano zinc (5.60 mm) and nano copper (4.78 mm). Among the different antibiotics tagmycin (14.13 mm) showed maximum inhibition zone followed by bacterinashak (10.92 mm). Copper oxychloride followed by nano zinc and tagmycin followed by bacterinashak, were found to be the most effective chemical and antibiotic treatments, respectively, for the *in vitro* management of *Pectobacterium carotovorum* subsp. *carotovorum* causing soft rot in carrot.

Keywords: *Pectobacterium*, chemicals and antibiotics, chemicals, antibiotics, bacterinashak

Introduction
Carrot (*Dacus carota* L.) is an important vegetable crop grown all over the world during winter in tropical and sub-tropical regions (Rubatzky *et al*., 1999) [1]. The edible part of carrot is being used as a vegetable for soups, stews, curries, pickles, salad and deserts. It is one of the rich sources of carotene in human diet (1890 µg/100 g fresh weight), precursor of vitamin A and high in fiber (Arscott and Tanumihardjo 2010; Nicolle *et al*., 2004) [2, 3].

Carrot is susceptible to several diseases such as Alternaria leaf blight, bacterial leaf blight, cavity spot, *Cercospora* leaf spot, cottony rot, damping off, powdery mildew, root-knot nematode and bacterial soft rot. Among these, bacterial soft rot is the most serious disease, characterized by soft, watery, and slimy decay of the taproot (Akbar *et al*., 2015) [4]. The disease typically infects the storage tissues of vegetables, with infection beginning in the field, while symptoms usually appear during storage (Agrios, 2006) [5].

In general, chemical substances are not recommended for managing bacterial diseases due to their potential risks to human health and negative impacts on the environment. However, several researchers have evaluated different chemicals for controlling bacterial soft rot, and among them, copper-based compounds have been found to be the most effective against Pectobacterium carotovorum subsp. carotovorum under *in vitro* conditions (Rashid *et al*., 2013) [6]. Copper based chemicals considerably reduced the infection rate, yield loss and percentage of disease reduction against *Pectobacterium carotovorum* subsp. *carotovorum* (Rahman *et al*., 2012) [7]. The antibiotics had a substantial effect on plant pathogenic microorganisms which formed cell wall degrading enzymes (Alice and Sivaprakasam, 1995) [8]. In view of the significant economic losses caused to carrot crops, the present study was conducted to isolate and identify the causal organism and to evaluate the efficacy of various chemicals and antibiotics against *Pectobacterium carotovorum* subsp. *carotovorum* under *in vitro* conditions.

2. Materials and Methods

The experiment was conducted in the Plant Pathology Laboratory, College of Agriculture, UAS, Raichur. It involved the purification and identification of the pathogen responsible for soft rot in carrot, along with the evaluation of different chemicals *viz*., copper oxy chloride, copper hydroxide, nano copper and nano zinc and antibiotics like, tagmycin, bacterinashak, kasugamycin, validamycin were tested against the growth of *P. carotovorum* subsp. *carotovorum.* Chemicals were evaluated at 500, 1000, 2000 and 3000 ppm concentration and antibiotics were evaluated at 50, 100, 200, 250 and 300 ppm concentrations for their efficacy against the growth of *P. carotovorum* subsp. *carotovorum* by following the well diffusion method.

2.1 Isolation and purification of the pathogen from infected carrot root

The soft rot infected carrots were collected from market area of different districts of Karnataka (Plate 1a). For isolation of the pathogen, small portion of diseased tissue was crushed using pestle and mortar aseptically using sterile distilled water, the suspension was left for few min undisturbed followed by serial dilution up to 10^{-5} and 10^{-6} and 0.1 ml of 10^{-5} and 10^{-6} solution was spread over on nutrient agar by pour plate technique. The plates were incubated at 30 °C for 24 hrs single colonies obtained were further purified using NA medium (Vimal and Anuradha, 2013) [9].

Plate 1a: Soft rot infected carrot sample

2.2 The bacteria identification

The bacteria were identified by the following tests

2.2.1 Grams staining reaction

A well isolated young colony was smeared on a glass slide followed by heat fixation. After a series of Grams staining reaction, described by Salle (1995) [10] and Schaad (1992) [11] at 100x magnification the slide was viewed.

2.2.2 Biochemical Tests

Various biochemical tests were performed. In the KOH solubility test, a bacterial colony was mixed with a 3% KOH solution, and changes in the consistency of the mixture were observed and recorded (Suslow *et al*., 1982) [12]. For the catalase test, 2-3 drops of freshly prepared 3% H_2O_2 (hydrogen peroxide) were placed on a glass slide containing a smear of pure bacterial culture, and the formation of bubbles within a few seconds was observed to determine catalase activity (Schaad, 1992) [11]. In lactose reduction test, the pathogen was inoculated to nutrient broth containing lactose disc and observed the color changes in test tubes (Kovacs, 1956) [13]. Nutrient agar containing 5 per cent gelatin with bacterial *Pcc* culture incubated at 30 °C for gelatin liquefaction test, 1-2 days followed by 5 °C in refrigerator for 15 minutes for and it was observed whether the bacteria liquefied gelatin or not (Salle, 1995) [10]. In the starch hydrolysis test, a pure bacterial culture was spot-inoculated at the center of a nutrient agar plate containing 1% soluble starch and incubated. After incubation, the plate was flooded with Lugol's iodine solution, and the presence or absence of clear zones around the colony was recorded (Cowan, 1974) [14].

2.2.3 Studies on pathogenicity

For proving pathogenicity, the surface disinfected carrots were injected with 250-300 µl (1×10^8 cfu/ ml) of bacterial suspension aseptically with the help of sterilized syringe. The crown portion was inoculated then placed in plastic cover with wet cotton and was incubated for three to four days for symptom expression.

2.3 *In vitro* management of *Pectobacterium carotovorum* subsp. *carotovorum*

Well diffusion method

Using the pour plate technique, a bacterial suspension (10 ml per 1000 ml of nutrient agar) was mixed with molten nutrient agar, and 15-20 ml of this mixture was poured into sterilized Petri plates. After the medium solidified, wells were created in the seeded agar using a cork borer and filled separately with the specified chemicals at the desired concentrations. The treated plates were then incubated at 30±1 °C for 96 hours, and the inhibition of the pathogen around each well was measured in millimeters.

2.4 Data analysis

The data collected during the experimental period were tabulated and analyzed using SPSS software, with a significance level set at $p<0.05$.

3. Results and discussions

3.1 Identification of bacteria from colony morphology

The colonies of *P. carotovorum* subsp. carotovorum on nutrient agar were observed to be creamy white, round, slightly raised, smooth with entire edges, and ranged from small to moderately large in size (Plate 1b). Corresponding types of colonies were reported by Dipak *et al*. (2013) [16].

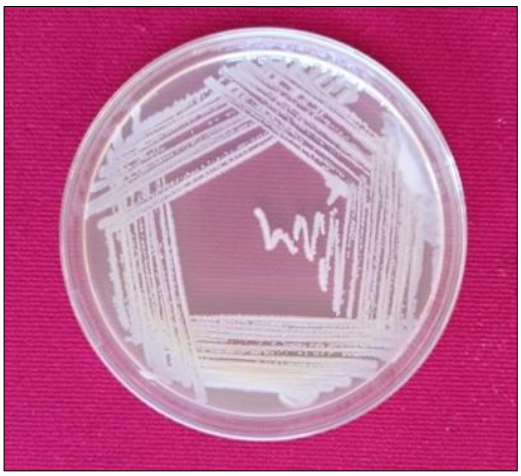

Plate 1b: Culture of *Pectobacterium carotovorum subsp. carotovorum*

3.2 Identification of bacteria from biochemical characters

The isolated bacteria, *P. carotovorum* subsp. *carotovorum* were confirmed by different biochemical tests (Table 1). The bacteria were gram negative as they resulting in red color cells after a series of Gram reaction test. In KOH solubility test, the bacteria formed a mucoid strand when lifted with the help of toothpick (Hind *et al*., 2015 and Sangeetha *et al*., 2020) [17, 18].

Table 1: Characteristics of isolated bacteria *P. carotovorum* subsp. *carotovorum* for various tests

Test	Reaction
Gram staining	+
KOH solubility	+
Catalase test	+
Lactose reduction test	+
Gelatin liquefaction	+
Starch hydrolysis	+
Pathogenicity test	+

The bacteria produced bubbles, indicating a positive catalase test. In the lactose fermentation test, the bacteria changed color from red to yellow (Rahman *et al*., 2012) [7]. Bacteria liquefied gelatin proves the test of gelatin liquefaction test (Thiyagarajan, 2016) [15]. Starch hydrolysis test confirms that hydrolyzed zone was not appeared around the colony, upon the inoculation of pathogen did not produce any amylase enzyme (Bradbury, 1986) [19].

3.3 Pathogenicity test

The pathogenicity of *P. carotovorum* subsp. *carotovorum* was proved on carrot by producing brown lesions development on carrot followed by rotting indicating the aggressive nature of the pathogen macerating the tissue emitting foul odor under favorable conditions which is attributed to the production of extracellular enzymes (Barbara *et al*., 1992). Based on morphological, biochemical and pathogenicity tests, the pathogen was identified as *P. carotovorum* subsp. *carotovorum*.

3.4 Efficacy of chemicals against *Pectobacterium carotovorum* subsp. *carotovorum*

Results indicated that among the antibacterial chemicals significantly superior efficacy was exhibited by copper oxychloride with an inhibition zone of 9.68 mm. Whereas moderate inhibition was observed by nano zinc (5.60 mm), nano copper (4.78 mm) and copper hydroxide (4.77 mm) which were on par with each other (Table 2 and Plate 2).

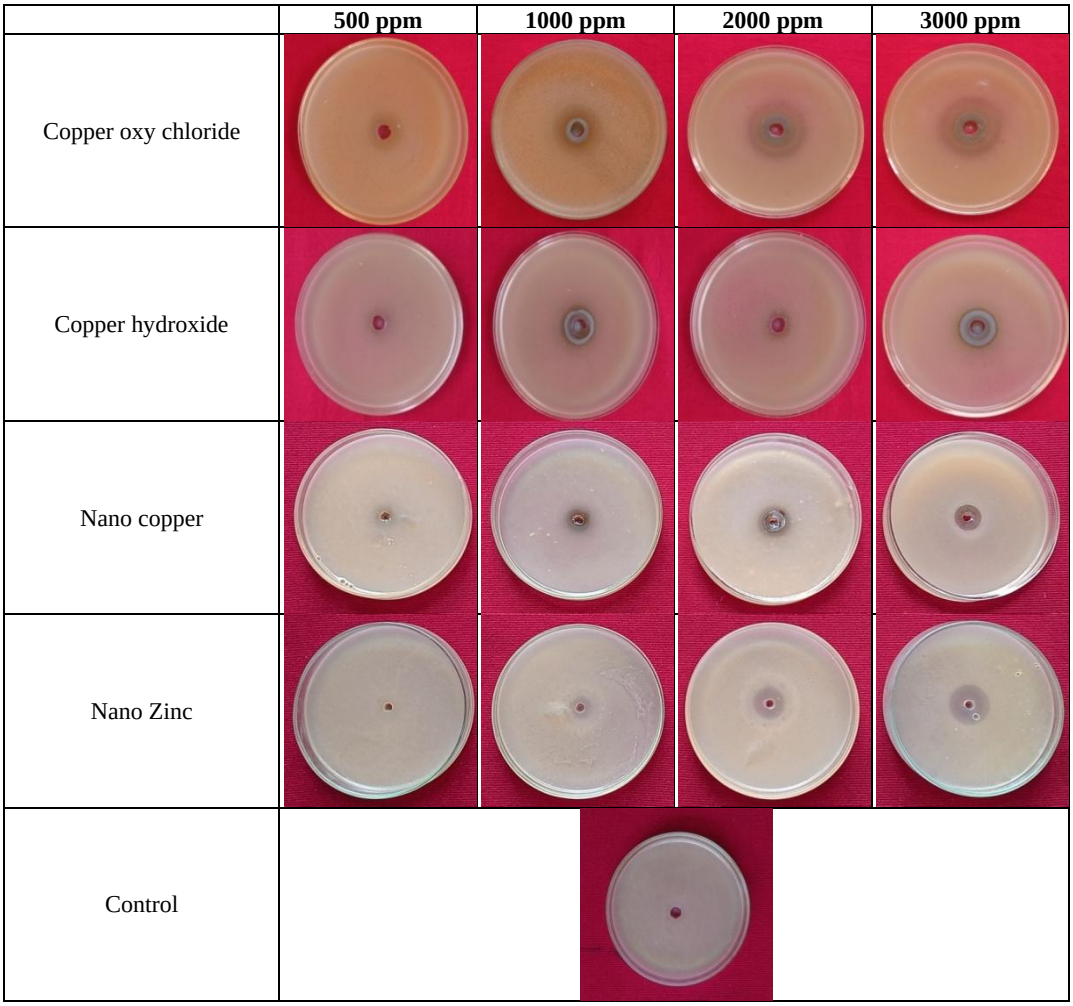

Plate 2: Bioefficacy of antibacterial chemicals against *Pectobacterium carotovorum* subsp. *carotovorum*

Table 2: Bioefficacy of antibacterial chemicals against *Pectobacterium carotovorum* subsp. *Carotovorum*

SL. No.	Chemicals	Mean Inhibition Zone (mm)				
		Concentration (ppm)				
		500	1000	2000	3000	Mean
1	Copper oxy chloride	5.32 (2.39)	9.99 (3.23)	11.49 (3.46)	11.94 (3.52*	9.68 (3.19)
2	Copper hydroxide	2.16 (1.63)	3.49 (1.99)	6.88 (2.71)	6.55 (2.65)	4.77 (2.29)
3	Nano copper	3.50 (2.00)	4.33 (2.19)	5.38 (2.42)	5.94 (2.53)	4.78 (2.28)
4	Nano zinc	3.75 (2.06)	4.77 (2.29)	5.34 (2.41)	8.55 (3.00)	5.60 (2.46)
5	Control	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)
	Factor	Chemicals (A)	Concentration (B)	A x B		
	S.EM ±	0.06	0.05	0.13		
	CD at 1%	0.19	0.17	0.40		

* Figures in the parenthesis are square root transformed values

The efficacy of each antibacterial chemical increased significantly with rising concentrations. Among all treatments, the interaction between antibacterial chemicals and their concentrations showed that copper oxychloride produced the largest inhibition zone of 11.94 mm at 3000 ppm, which was significantly higher than all other chemicals tested, followed by the same chemical at 2000 ppm (11.49 mm), 1000 ppm (9.99 mm), and 500 ppm (5.32 mm). Copper compounds interact with various chemical groups within cells, especially thiol groups, leading to non-specific denaturation of proteins and enzymes. Additionally, copper ions can disrupt the pyruvate dehydrogenase system, inhibiting the conversion of pyruvate to acetyl-CoA in mitochondria. Copper also reacts with essential cellular elements and cell surface ligands, which can interfere with membrane function (Warnes *et al*., 2010; Grass *et al*., 2011 and Chaturvedi and Henderson, 2014) [21-23].

The next most effective antibacterial chemical was nano zinc, showing inhibition zones of 8.55 mm (3000 ppm), 5.34 mm (2000 ppm), 4.77 mm (1000 ppm), and 3.75 mm (500 ppm) from higher to lower concentrations. Among all treatments, the lowest efficacy was observed with copper hydroxide at 500 ppm, producing a zone of inhibition of 2.16 mm.

Research findings on the efficacy of antibacterial chemicals are in accordance with Yulia *et al*. (2020) [24], who reported that, copper oxychloride and copper hydroxide were effective inhibiting the growth by 10.5 mm and 17.5 mm respectively against *Pectobacterium* spp. causing soft rot of tomato. Similarly, the findings can also be well comparable with the studies conducted by Thammaiah *et al*. (2005), who obtained copper oxychloride as significantly effective against the pathogen *E. carotovora* subsp. *carotovora* at 3000 ppm concentration. Six chemical substances were evaluated (Akter and Sultana, 2018) against *E. carotovora* under *in vitro* conditions *viz*., Copper oxychloride (0.2%), Mancozeb (0.2%), Boric acid (0.1%), Kasugamycin (0.02%), Carbendazim (0.3%) and Sodium hypochlorite (0.2%) were tested by well diffusion method. Maximum zone of inhibition was obtained with copper oxychloride (30.35 mm) followed by Mancozeb (20.15 mm). Thiyagarajan (2016) [15] evaluated various chemicals against *E. carotovora* subsp. *carotovora*, the causative agent of tip-over disease in banana, and reported that copper hydroxide showed the highest inhibition (39.44 mm), followed by copper oxychloride (37.77 mm), while bleaching powder exhibited the lowest inhibition (15.60 mm).

3.5 Bioefficacy of antibiotics against *Pectobacterium carotovorum* subsp. *carotovorum*

Among the antibiotics tested, Tagmycin exhibited significantly superior efficacy with a mean inhibition zone of 14.13 mm. Bacterinashak (10.92 mm) and Plantamycin (8.00 mm) showed moderate effectiveness, whereas Kasugamycin exhibited the least inhibition (1.16 mm) and Validamycin showed no inhibition against the pathogen (Table 18, Plate 16). The interaction effect of antibiotics indicated that Tagmycin was significantly effective at all tested concentrations, with inhibition zones of 20.10, 16.03, 12.90, 11.28, and 10.33 mm at 500, 300, 250, 100, and 50 ppm, respectively, outperforming all other treatments. Whereas bacterinashak was next best effective antibiotics which is moderately effective in arresting the growth of the bacterium and significantly least efficacy was exhibited by kasugamycin at all the concentrations, whereas validamycin did not show even at higher concentration of 500 ppm (Table 3 and Plate 3). This effect may be attributed to the ability of the antibiotic to interfere with bacterial protein synthesis. By binding to the ribosome, it inhibits translation initiation through direct competition with the initiator transfer RNA, ultimately killing the pathogen. Streptomycin, a known protein synthesis inhibitor, binds to the 16S rRNA of the 30S ribosomal subunit, disrupting the attachment of formyl-methionyl-tRNA. This interference causes codon misreading, inhibits protein synthesis, and eventually leads to the death of microbial cells (Mahmood *et al*., 1981) [27].

The findings of the present study are supported by previous reports. Farag *et al*. (1984) [28] observed that *E. carotovora* was sensitive to antibiotics such as streptomycin, tetracycline, ampicillin, and chloramphenicol (Mazzucchi and Svampa, 1972) [29]. Similarly, Thiyagarajan (2016) [15] reported that Streptocycline significantly inhibited the growth of *E. carotovora* subsp. carotovora *in vitro*, with a mean inhibition zone of 53.90 mm, followed by K-cycline (50.00 mm), while Bacterinashak showed comparatively lower efficacy, with an average inhibition zone of 42.30 mm.

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Table 3: Bioefficacy of antibiotics against *Pectobacterium carotovorum* subsp. *carotovorum*

SL. No.	Antibiotics	Mean Inhibition Zone (mm)					
		Concentration (ppm)					
		50	100	250	300	500	Mean
1	Bacterinashak	6.53 (2.63)	8.26 (2.94)	10.65 (3.33)	12.49 (3.59)	16.67 (4.14)	10.92 (3.37)
2	Kasugamycin	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	4.66 (2.23)	1.16 (1.28)
3	Plantamycin	5.46 (2.42)	7.58 (2.82)	8.27 (2.95)	9.32 (3.12)	9.36 (3.14)	8.00 (2.91)
4	Tagmycin (*Streptomycin* sulphate)	10.33 (3.28)	11.28 (3.42)	12.90 (3.65)	16.0.3 (4.06)	20.10 (4.53)	14.13 (3.82)
5	Validamycin	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)
6	Control	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)
	Factor	Antibiotics (A)	Concentration (B)	A x B			
	S.EM ±	0.06	0.05	0.13			
	CD at 1%	0.17	0.16	0.39			

* Figures in the parenthesis are square root transformed values

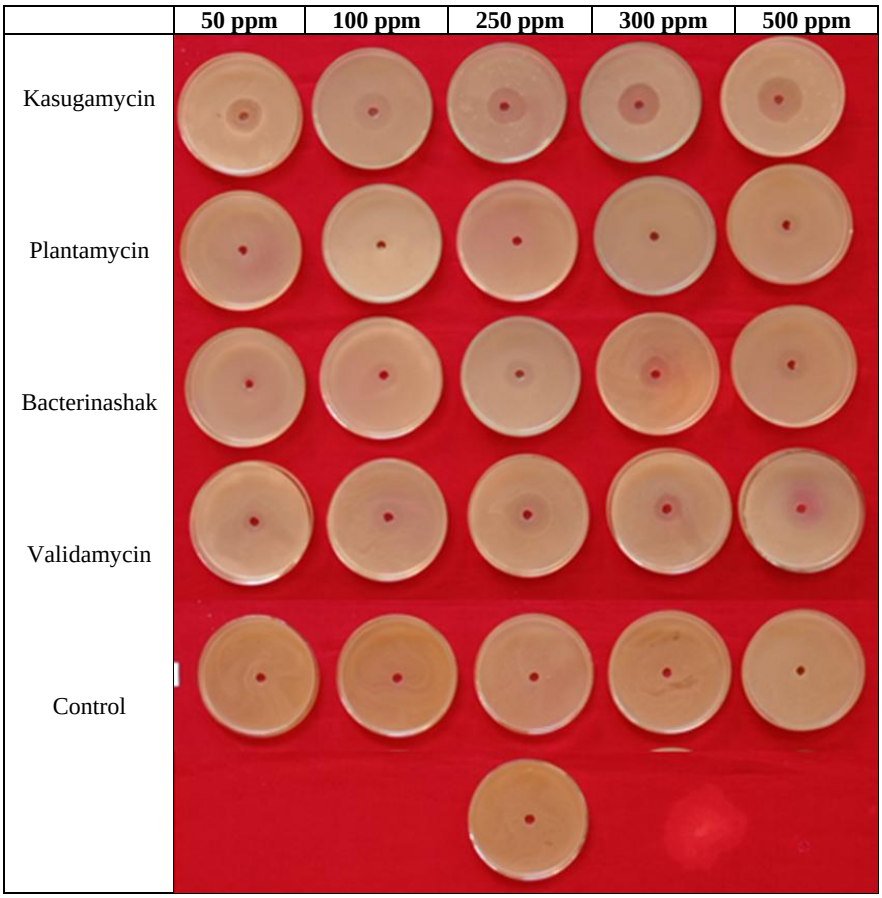

Plate 3: Bioefficacy of antibiotics against *Pectobacterium carotovorum* subsp. *carotovorum*

Conclusion

In vitro studies on the efficacy of antibacterial chemicals and antibiotics against *P. carotovorum* subsp. *carotovorum* revealed that, among the chemicals tested, copper oxychloride produced the largest inhibition zone, followed by nano zinc. Among the antibiotics, Tagmycin was found to be significantly more effective than the others, followed by Bacterinashak, while Kasugamycin exhibited poor efficacy. These results suggest that these chemicals could be utilized for managing soft rot of carrot, a major threat to carrot cultivation. However, comprehensive and large-scale research is needed to identify more effective methods for controlling this disease.

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