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THE EFFECT OF PYRIDINIUM CHLOROCHROMATE (P.C.C.) ON THE BACTERIAL LOAD OF CHILLI PEPPER (*Capsicum annuum*) SEEDS AND SEEDLINGS

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ABSTRACT
The antibacterial activity of the synthetic compound, Pyridinium Chlorochromate was tested with four bacterial isolates designated GBB418, GBB420, GBB421 and GBB422 obtained from infected pepper plants. Tissue tolerance concentration of the compound was evaluated by soaking the seeds in different concentrations of the compound for 30 minutes and percentage germination was calculated. Simulated infection was done by soaking the seeds in a concentration above the Minimum Inhibitory Concentration (MIC) value for 30 minutes and also the standardized organism was allowed to act for 30 minutes on the already soaked seeds and the residual bacterial loads were estimated daily. A study of the compound Pyridinium Chlorochromate (PCC) shows its antibacterial effect thus, permits the germination of pepper seeds when soaked with it.Moreso, the compound also cause a reduction in the bacterial load of pepper seeds.

KEYWORDS: Pyridinium Chlorochromate, *Capsicum annuum,* Tissue tolerance concentration, Simulated infection.

INTRODUCTION

Pyridinium Chlorochromate or PCC, $C_5H_5NHCrO_3Cl$ is a reddish orange solid reagent used as an oxidant to oxidize primary alcohols to aldehydes and secondary alcohols to ketones. It is produced by the reactant mixtures of a molecule of pyridine,chromic acid and concentrated hydrochloric acid (Corey and Suggs,1975). Pepper (*Capsicum annuum* L., Solanaceae) is an annual or biannual herbaceous plant that is found in tropical America, Korea, Japan, India and Nigeria. It flowers from May to June and the fruits ripen from July to September. The ripen fruits are red and 5-10 cm in length (Kim, 1999; Berke and Shieh, 2001). *Capsicum* species popularly known as pepper is the world's second most important crop after tomato (Yoon *et al*., 1989). According to Bosland (1994) the genus *Capsicum* belongs to the family Solanaceae. Cobley and Steele (1976) reported that apparently between 5200 and 3400 BC, the native of Americans were growing *Capsicum*, which places it among the oldest cultivated crops. According to Adamu *et al*. (1994) Nigeria is the largest producer of pepper in Africa, accounting for about 50% of the African production. Five different species were domesticated; *Capsicum annuum, C. frutescens, C. baccatum, C. Chinese* and *C. pubescens.* Among these species*, C. annuum* is the most widely spread and most important. The plant is a herbaceous annuals usually growing from 45cm – 65cm high. In Nsukka yellow pepper (which is characterized by its yellow colour at fruit ripening and a unique aroma), 1 – 3 fruits do occur in the axil of one leaf (Amako, 1994). Consumption of pepper accounts for about 20% of the average vegetable consumption per person per day in Nigeria (Erinle, 1989; Alegbejo, 2002).

It is used extensively in food flavouring in the daily diet of over 120 million Nigerians irrespective of their socio-economic status. It is used in the preparation of soup and stew, which are among the major essential compliments of staple based on cereals and root crops and also forms remedies for toothache and sore throat, (Leung and Foster, 1996; Bosland, 1994). The capsaicin extract from sweet pepper is used in pharmaceuticals as a counter irritant balm (Purseglove, 1997). In Japan and China, it is used topically in an ointment form to treat myalagia (But, 1997) and in Germany; it is approved as a topical ointment for the relief of painful spasm. Capsicum is regarded as a neuropathetic pain reliever, therefore is used in the treatment of diabetic neuropathy and also in the management of surgical neuropathic pain in cancer patient (Messiaen, 1992). In Korean traditional medicine, the fruit of the red pepper is referred to as Namcho , and has pharmaceutical effects such as Onjoong , to warm the coldness Kaewui , to activate the stomach function Sosikche, to smooth blocked internal organs and is used to treat stomach-aches, emesis, dysentery, chilblain, and scabies. The stem is called Nalchogyung and is used to treat rheumatic psychroalgia. The root, called Nalchodoo , is used to treat asthenia of the limbs (Souka, 1985; Chung and Shin, 1998). The leaves are also used to treat emesis, dysentery, and scabies (Souka, 1985; Chung and Shin, 1998), and they have been shown to have anti-mutagenic anti-cancer, anti-microbial, anti-lipid peroxidation, and anti-complementary activity (Park *et al*., 1992; Park *et al*., 1997; Chung and Shin, 1998; Ra *et al*., 2002; Kim *et al*., 2003).

On this premise, the aim of this study was to investigate the antibacterial effect of PCC on isolates from infected pepper plants,determine the concentrations of the compound that will permit the germination of the seeds and seed protection during storage.

FIGURE 1. Chili peppers are characterized by the large diversity of fruit types according to color, shape, fruit thickness, pungency, and taste. Based on particular taste preferences and adaptability, varieties have been selected for production in specific regions around the world.

MATERIALS AND METHODS

Organisms Source and Culture Conditions

The bacterial isolates of chilli peppers (*Capsicum annuum*) used were obtained from the culture preserved on Nutrient agar slant (coded GBB 418; GBB 420; GBB 421 and GBB 422) that has been previously isolated from diseased pepper plants by an M.Sc student in the Department of Microbiology, Obafemi Awolowo University Farm Ile-Ife,Osun- state,Nigeria. The pepper seeds were obtained from National Horticultural Research Institute,Idi-Ishin (NIHORT), in Ibadan, Oyo-state,Nigeria. The seeds germinate in 6-10 days, they are light, about 140 per grams, and retain their viability for 2-3 years.

PREPARATION OF INOCULUM

Harvesting of the sub-cultured in the Nutrient Broth

All the organisms sub-cultured in the broth were harvested by centrifuging at about 3500 rpm for 15 minutes. The supernatant was decanted and equal volume of sterile normal saline (0.85%w/v) was added and washed again thrice (by resuspending the pellet and centrifugation).

Standardization of Inoculum

The cultures from fresh incubated inoculum were centrifuged at 3,500 rpm for 15 minutes, washed and resuspended in 0.85% Nacl (w/v). The cultures were standardized to O.D. values of 0.07 using VC Visibe Double Beam, spectrophotometer for CE 7450,7000 series at 540nm wave length transmitter.

Viability test of the Seeds

This is the testing used to know the potency, efficiency and viability of a particular type of seeds. Two Petri-dishes were washed inside of which was a filter paper each and they were soaked with distilled water and then sterilized using the autoclave.After surface sterilization of the seeds with sodium hypochloride, one of the dishes was later seeded with the Ife-seed type (50) and the other the NIHORT seed (50), Ibadan.

EFFECT OF PYRIDINIUM CHLOROCHROMATE ON VIABILITY

Percentage germination was determined at different concentrations of the synthetic compound. Here, 30 surface sterilized seeds were soaked for 30 minutes in the synthetic compounds at different concentrations carried out with more than one concentration i.e. using two dishes having the same concentrations for comparison. After appropriate commencement for germination, the numbers of germinated seeds were observed for the same concentration and percentage viability was estimated.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC)

The micro-pipette used was graduated to take 50 microliters (?l) of fluid at a time. With this all the 96 wells of the sterile micro-titres plates were first pipetted with 2 drops of Nutrient broth (double strength) to each well horizontally. Each well of the micro-titre plate can accommodate up to 200 ?l of fluid. This was followed by 50 ?l of different concentration of the synthetic compounds to all the wells except the drug control well, and the 50 ?l of the standardized organism in the entire well except the culture or organism control well. In addition to this, 50 ?l of normal saline were pipetted also into both the organism control and the drug control respectively. The plates were incubated at different temperature, one at 37^{o}C for 16-24 h, the second at the room temperature between 25 - 27^{o}C for 16-24 h which later were compared. At the end of the incubation period, the Minimum Inhibitory Concentration (MIC) can be read as the concentration of the compounds or drugs that prevent growth of the organism. Growth was determined by the turbidity of each well after necessary incubation period. The Minimum Bactericidal Concentration was determined by sub-culturing from the wells where there was no visible growth into sterile Nutrient agar and incubates at both 37^{o}C and at room temperature for comparison, for 16-24 h. The lowest concentration that prevents growth after necessary period of incubation is the Minimum Bactericidal Concentration (MBC).

TISSUE TOLERANCE CONCENTRATION (TTC)

Filter papers were place inside the Petri-dishes and then sterilized using the autoclave, 30 seeds were counted and then steeped for 30mins with different concentration of the compound (Pyridinium Chlorochromate).The seeds were then transfer into the already sterilized petri-dish in duplicate and then were labeled with the appropriate concentration used. A control was also set up without steeping inside the synthetic compound. The germination was then monitored and the numbers of seeds that germinate were being counted daily. The experiment eventually stopped after all the viable seeds in the dishes have completely germinated. The percentage germination was calculated, also the lengths of the radicle and shoot were measured with the graduating ruler in centimeter (cm).The purpose of this experiment is to determine the concentration of the compound that will permit the germination of the seed.

SIMULATION OF INFECTION

The pepper seeds (30) were counted and weighed. Thereafter, the seeds were surface sterilized and then, transferred into a clean beaker. 1 ml of the compound at the concentration, a step above MIC was added to the seeds and then allowed to steep for 30 minutes. At the end

of this time, the seeds were removed aseptically (the remaining volume of the compound were measured) and then transferred into a sterile test tube.1 ml of the standardized organism suspension was added and then allowed to soak the seed for 30 minutes. The contents of the test tube were shaken at intervals to allow even distribution of the organisms. At the end of the period, the seeds were removed aseptically and then transferred into a sterile Petri-dish that has filter paper inside. Also, the remaining volume of the organism was measured. At day 1, two seeds were removed from the Petri-dish into 10ml of sterile distilled water, shaked very well and 3 fold serial dilutions was made from it. From the 3 fold dilution (10^{-3}), 0.5 ml of the dilution was taken aseptically into a Petri-dish and the already melted and cooled nutrient agar was added to it and mix together to attain uniformity (pour plate method). After setting, it was incubated at room temperature for 24 h. This was done for; organism + seed + compound; and for the two control i.e. organism + seed control and the seed control only, all being carried out in duplicate. At the end of the incubation, organisms were counted and correlated. Two seeds removed from each plate daily until the seed germinates. This experiment is a demonstration of possible application of the compound for seed protection during storage and prior to planting.

RESULTS AND DISCUSSION

For the four isolates used for the study as GBB 418, GBB 420, GBB 422 and GBB 421, the MIC range is 200-250 ?g/ml for GBB 420, GBB 421, and GBB 422; 100-150 ?g/ml for GBB 418;while the MBC ranged from 250-300 ?g/ml for GBB 420, 400-500 ?g/ml for GBB 422 and 300-400 ?g/ml for GBB 421 (Table 1), respectively.

TABLE 1. Determinations of MIC and MBC of Pyridinium Chlorochromate against bacterial isolates from infected pepper seed

ISOLATE CODE	MIC (?gml^{-1})	MBC(?gml^{-1})
GBB 421	200-250	300-400
GBB 422	200-250	400-500
GBB 420	200-250	250-300
GBB 418	100-150	150-200

MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; Drug Used, Pyridinium Chlorochromate; ?, micro

TABLE 2. Effect of different concentrations of PCC on germination of pepper seeds

Days	Number of germinated seeds				
	Treated seeds at different concentrations of Pyridinium Chlorochromate(?gml^{-1})				
Day	Control Seeds	1000	500	400	300
0-8	NG	NG	NG	NG	NG
9	2	0.5	1.0	1.0	NG
10	3	0.5	1.0	2.0	NG
11	3	0.5	1.0	3.5	1
12	5	0.5	1.0	5.0	1
13	5	1.0	1.0	6.0	2
14	9	2.0	1.5	6.0	2.5
15	11	3.5	2.5	7.5	3.5
16	13	5.5	4.5	8.5	4.5
17	13	9.0	5.5	10.0	6.5
18	15	11.0	7.0	12.0	8.0
19	15	13.0	8.0	14.5	10.5
20	15	14.5	10.0	15.5	11.5
21	16	16.5	12.0	16.5	13.0
22	16	17.5	14.0	18.0	14.0
23	17	19.0	15.0	19.0	15.0
24	19	19.5	16.5	19.0	16.0
25	21	20.5	18.5	20.5	18.0
26	22	20.5	18.5	20.5	18.5
27	23	21.0	20.5	21.5	20.0
28	23	21.5	22.0	21.5	20.0
29	25	21.5	23.5	23.0	21.0
30	26	21.5	24.0	24.0	21.0

Values represent average of two means; NG,No Growth;?, micro

Thus, the comparative antibacterial effect of the compound in terms of the MIC was GBB 418 >GBB 420 > GBB 422 > GBB 421. Similar observation has been previously reported by Babalola (1998). For the fact that bacterial cells are not constant in their sensitivity to antimicrobial agent and their responses differ from one drug to another as a result of certain irregularities, hence standardization of their inoculum to be used is necessary.The Minimum Inhibitory Concentration (MIC) varies widely with size of inoculum. For example, a strain may appear to be fully sensitive when treated with a small inoculum but when heavy inoculum is used, the MIC may

rise to concentration unattainable when normal doses are given (Strokes, 1975). It shows that the bactericidal concentration to kill an organism may not necessary kill the other organism therefore varies in concentration. From these result, it can be said that the compound is effective in killing these bacterial isolated from diseased plants. For the tissue tolerance concentration (TTC), it would appear that the seeds were able to tolerate the compound even up to the concentration of 1000 ?g/ml. For instance, as the concentration increases, the average number and percentage of seed germination increases but not appreciably initially, especially at concentration 500 ?g/ml (Table 2 and 3).

TABLE 3. Percentage Germination at different concentrations of PCC on Treated and Untreated Seeds

	percentage of germinated seeds				
	Treated seeds at different concentrations of Pyridinium Chlorochromate (?gml^{-1})				
Day	Control	1000	500	400	300
	Untreated Seeds				
0-8	NG	NG	NG	NG	NG
9	6.67	1.67	3.33	3.33	NG
10	10.00	1.67	3.33	6.67	NG
11	10.00	1.67	3.33	11.67	3.30
12	16.67	1.67	3.33	16.67	3.30
13	16.67	3.33	3.33	20.00	6.67
14	30.00	6.67	5.00	20.00	8.30
15	36.67	11.67	8.30	25.00	11.67
16	43.30	18.33	15.00	28.3	15.00
17	43.30	30.00	18.30	33.30	21.67
18	50.00	36.67	23.30	40.00	26.67
19	50.00	43.30	26.67	48.30	35.00
20	50.00	48.30	33.30	51.67	38.30
21	53.30	55.00	40.00	55.00	43.33
22	53.30	58.3	46.67	60.00	46.67
23	56.67	63.30	50.00	63.30	50.00
24	63.30	65.00	55.00	63.30	53.30
25	70.00	68.30	61.67	68.30	60.00
26	73.30	68.30	61.67	68.30	61.67
27	76.67	70.00	68.30	71.67	66.67
28	76.67	71.67	73.30	71.67	66.67
29	83.30	71.67	78.30	76.67	70.00
30	83.30	71.67	78.30	80.00	70.00

Values represent average of two means; NG,No Growth;?, micro

TABLE 4. Length and Shoot Dimension at different concentrations of PCC on Treated and Untreated Seeds

	Treated seeds at different concentrations of Pyridinium Chlorochromate (?gml^{-1})									
Germinated Control Seed		1000		500		400		300		
LR	LS	LR	LS	LR	LS	LR	LS	LR	LS	
3.439	1.32	2.96	1.41	3.26	1.45	3.44	1.28	3.52	1.38	

Values represent average of two means; ?, micro; LR,Length of the root (cm); LS, Length of the shoot(cm)

However, the onset of germination was delayed and varies from one concentration to another. A germination takes off was fasted at 400 ?g/ml and least for the 300 ?g/ml concentration starts germinating which was the 11th days. Furthermore, the dimension, length (cm) of the roots and shoots of the chilly pepper at different concentrations were measured using measuring ruler. Generally, the taproot was longer than the shoot. When compared with the untreated control, all treatments affect the root and shoot lengths but the effect was most pronounced between 300 ?g/ml and 500?g/ml in relation to the root and shoot respectively, and least pronounced at 400 ?g/ml and 1000 ?g/ml, especially the shoot and root where there was much difference (Table 4). It was also noted that the treated seedlings were stronger than the untreated control especially at 400 ?g/ml. This would suggest that the compound strengthened the seedlings, thus allowing them also stand more erect.The simulated infection results showed that there was a decrease in the number from 2.50 x 10^5 cfu/ml for GBB 418 (using 200 ?g/ml) to 1.10 x 10^5 cfu/ml on the 6th day for the infected and treated seeds (Table 5). For GBB 420 the same reduction was noticed from 2.80 x 10^5 cfu/ml to 1.30 x 10^5 cfu/ml (Table 6). This shows that the compound was effective in killing the bacterial with little absorption inside the seeds during the period of soaking. For the control experiment without treatment, an initial reduction was observed for the first two days (2.30 to 2.20) x 10^5 cfu/ml for GBB 418 and (2.50 to 2.40) x 10^5 cfu/ml for GBB 420. But by the third day, a gradual increase was observed. For GBB 418, the bacterial load increased from (2.20 to 2.50) x 10^5 cfu/ml and (2.40 to 2.70) x 10^5 cfu/ml for GBB 420. The initial

decrease may be due to the hypochloride that was used for surface sterilization of the seeds which also has antibacterial activity and thus kill some of the bacterial. But since the seeds did not absorb so much of the hypochloride only 3 minutes was used for surface sterilization and as the effect of the hypochloride was over there is an increase in the number of colonies forming unit.

TABLE 5. Bacterial Load of Treated and Untreated Infected Seeds

Sampling time (days)	Seed+Organism+Compound Treated ($x10^5$)	Seed+Organism Untreated ($x10^5$)
0	2.50	2.30
1	2.30	2.20
2	2.10	2.50
3	2.00	2.80
4	1.60	3.00
5	1.40	3.20
6	1.10	3.40

Isolate Used, GBB 418; Concentration of PCC used, 200?g/ml.

TABLE 6. Bacteria Load of Treated and Untreated Seeds

Sampling time (days)	Seed+ Organism + Compound Treated ($x10^5$)	Seed+Organism Untreated ($x10^5$)
0	2.80	2.50
1	2.60	2.40
2	2.40	2.70
3	2.20	3.00
4	1.80	3.20
5	1.60	3.40
6	1.30	3.60

Isolate Used, GBB 420 ; Concentration of PCC used, 300 ?g/ml.

CONCLUSION

This study demonstrated that the synthetic compound, Pyridinium Chlorochromate (PCC) has antibacterial effect on the organism isolated from infected pepper plants. Also, all the concentrations of the PCC prepared permit the growth of pepper seeds with appreciable percentage germination especially at concentrations between 400 and 500 ?g/ml. Moreover, the seedlings from the treated seeds were stronger and stood more erect than that of the control experiment that had no compound treatment resulting the compound by enhances the growth of the pepper seeds. The simulated infection experiment is a demonstration of possible application of the compound for seed protection during storage and prior to planting. In this way, pepper seedlings can be protected against bacterial diseases by soaking the seeds in the synthetic compound, Pyridinium Chlorochromate (PCC) before planting. On the other hand, the soaked seeds can be dried and packed in a suitable sack or nylon for protection during storage. More work can still be done to standardize the optimum conditions. Within the scope of this study, I will recommend concentrations of either 400 or 500 ?g/ml for soaking planting. This experiment can be studied or continued further by experimenting or carrying it out on the field to confirm this assertion. Finally, the compound Pyridinium

Chlorochromate (PCC) can be synthesized on a large scale acting as an effective antibacterial agent.

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METRIC SYSTEM

Length: cm (centimeter)
Volume: ml (milliliter);?l (microliter)
Weight: gm (gram); ?g (microgram)