

Use of Amino Oligosaccharins and Alternaria Fine Protein in the Management of Crown Gall Disease in Roses

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Abstract

Rose (*Rosa hybrida* Vill.) production is limited by a variety of factors such as poor mineral nutrition, high salinity, pests and diseases. Crown gall disease caused by *Agrobacterium tumefaciens* causes a significant damage to roses in Kenya. The study was carried out in Winchester farm (Nairobi) and Bahati farm (Nakuru) to determine the effects of a mixture of amino oligosaccharins and Alternaria fine proteins on crown gall disease in roses. The experiment was conducted on *Rosa hybrida* var. Mariyo in a Randomized Complete Block Design with four replications. The treatments comprised of different rates 0.5, 1, 1.5g/L of water of the product at 3% concentration applied as foliar spray and a commonly used product copper sulphate pentahydrate (Mastercop) produced by ADAMA applied at 2ml/L as the standard and water as a negative control. Crown gall tissues were collected from four different roses per treatment from the two sites, counted and used for biochemical tests in Kabete laboratory. Bacteria were isolated by culturing and the representative colony types growing on nutrient agar media selected and sub-cultured by successive streaking on nutrient agar media. The biochemical test for the different isolates was done to identify the bacterial isolates. Application of the mixture of amino oligosaccharins and Alternaria activated protein at the rate of 1.5g/L had significant effects on galls formation and reduced the numbers significantly at both farms. The gram reaction indicated that the selected isolates were gram negative and were positive for motility, catalase, oxidase, lactose, mannitol, and salt tolerance tests. There was a significant reduction in the number of galls and size following the application of amino oligosaccharins and Alternaria fine proteins as well as improvement in plant growth. The ability to manage the disease can be attributed to enhanced defense enzyme activity enhanced by amino oligosaccharins and Alternaria fine proteins.



Article History

Received: 07 April 2020

Accepted: 30 July 2020


Keywords:

Alternaria Fine Protein;
Chitosan;
Oligosaccharins;
Rose Shoots.

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Doi: <http://dx.doi.org/10.12944/CARJ.8.2.07>

Introduction

Globally, the Kenyan flower industry is the third major flower exporter by value and volume just behind the Netherlands and Colombia on a global level¹ while in Africa, it is the largest producer and the leading supplier of Fair trade flowers to Europe accounting for 50-60% of total volumes.² The flower production in the country is mainly large scale and export oriented.³ Cut flower is one of the two commodities where large-scale production dominates in the country and roses make up over 70% of all buds, trees, roots, flowers and foliage that is exported from Kenya.⁴ Roses alone account for about 35% of the total value of horticultural exports and therefore form the most important export product.⁵

In commercial production of roses, yield is restricted by an assortment of variables such as light, humidity, mineral nutrition, temperature, salinity and biotic factors.⁶ The common biotic factors in cut-flower roses include pests and diseases. Crown gall caused by *A. tumefaciens* causes a significant damage to roses in Kenya. The bacterium is found in the soil and it is responsible for the *tumorous* growth found in infected plants.⁷ The pathogen is widespread, naturally occurring soil bacterium that causes crown gall in numerous plant species in nurseries, commercial production and uncultivated areas and has the capacity to introduce new genetic material into the plant cell. Significant annual losses of between 5-6% are faced by the flower growers worldwide due to the disease in the form of lack of vigor, reduction in foliage, low quality rose flower, low productivity and increased susceptibility of infected plants to pathogens and environmental stress.⁸ In other instances, losses of between 10-30% in nursery stalk have been reported in fruit trees. Both yield loss and stunting of growth may occur when seedlings or young cuttings are infected in the early stages of plant growth.⁸ Many strategies have been used in the management of crown gall disease including the use of chemicals, pre-plant application of soil sterilants, soil solarization, use of herbicides and soil amendments.⁹ Despite losses incurred in flower exports due to effects of the disease and high residual effect of pesticides, research especially focusing on management of *A. tumefaciens* is limited. The use of oligosaccharins and alternaria fine protein has the potential to be one of the safest means to manage the plant disease. Oligosaccharins induce

responses that may help the plant to resist disease and have positive effects on growth and development while the alternaria fine protein accelerates plant growth vigor, increases proline content and cellulase strength. Therefore, this study sought to investigate effects of amino oligosaccharins and alternaria fine protein on crown gall disease of roses.

Materials and Methods

A-tailing is the mixture formulation using the active ingredient (A.I) Fine alternaria activated protein and amino oligosaccharins. The content of each active ingredient is three (3%) and the product is available in formulation specification as wettable powder (WP) and currently registered in China.

Experimental Materials, Design and Treatment Application

The experimental material used was *Rosa hybrida* var. Mariyo both at Winchester and Bahati farms. The variety was chosen because it's high yielding and has a continuous market as compared to other varieties whose market is seasonal.⁵ Its susceptibility to *Agrobacteria* made it appropriate for this research therefore. An existing infected crop was used for the research and was laid out in a randomized complete block design. Two-year-old rose of variety Mariyo planted in pumice (an inert growing media that is a by-product of volcanic ash) was pruned and sprayed at two weeks' intervals with oligosaccharins and fine Alternaria protein at various concentrations. The treatment concentrations comprised of a mixture of oligosaccharins and fine Alternaria protein at 3% concentration applied as foliar spray at 0.5, 1, 1.5g per litre of water. Commonly used product in the market, Mastercop, was applied at 2ml/L and was used as the standard and a negative control. Re-application of the treatments was done at fortnight intervals. Six plants from each plot were randomly selected from each treatment and used for data evaluation.

Collection of Crown Gall Samples

Ten galls of diameter 3-10cm were collected and put in sterile bags from two different rose farms (Winchester farm and Bahati roses) located in Nairobi and Nakuru, respectively. The samples were labeled and transferred under a cool box to the laboratory in Kabete for isolation.

Determination of size of galls

Size of *Agrobacteria* galls formed on rose plants were measured and recorded fortnightly. This was used for disease severity determination.

Isolation of Bacteria from the Gall

Agrobacteria gall samples from treated and untreated plots having young tumors were transferred to the laboratory for isolation. Small fragments of tumor tissue were chopped and crushed with sterile scalpel blade in a few drops of sterile distilled water. The suspension was then left to stand for approximately 15 min and 100 μ L was spread with a loop onto the nutrient agar (NA), which contains 0.5% peptone, 0.3% beef extract, and 1.5% agar, and incubated at 28°C. Representative colony types growing on nutrient agar media were selected from each gall sample by use of a sterilized wire loop and sub cultured by successive streaking on new nutrient agar media.

Disease Assessment

Rose plants were randomly inspected for the assessment of crown gall disease using disease incidence and severity levels. Disease incidence was determined using the formula by.¹⁰

$$\text{Disease incidence} = (\text{Number of infected plants}) / (\text{total number of plants}) \times 100$$

While severity was assessed based on the size of the infection where mild; 1-3 cm, moderate; 4-6 cm and severe; above 6 cm.

Characterization of *Agrobacterium tumefaciens* Biochemical Test

Biochemical test for the distinctive isolates was carried out following the Bergey's manual of Determinative Bacteriology.¹⁰ Motility, gram stain, catalase, oxidase production, utilization of lactose, mannitol and salt tolerance (2%) tests were conducted.

Gram Staining

Gram staining was done by making bacterial smears from two days old cultures on sterile microscope slides. The smears were air-dried and then after, heat fixed by passing the slides over a flame and then Gram stained. The slides were observed under

a compound microscope at magnification of $\times 1000$.

Utilization of Lactose

Phenol red mannitol broth with 1% lactose was used. The broth contains peptone, phenol red(indicator) a Durham tube and carbohydrate(lactose). Each test tube was aseptically inoculated using an inoculating loop and incubated at 37°C for 24 hours. A positive result was indicated by gas production (bubbles) on inverted Durham tube.

Motility Test

A semi solid agar medium was prepared in a test tube and inoculation done by use of a wire loop to make a single stab at the centre of the tube. Incubation at 37°C (conditions favoring motility) were carried out and examined at intervals (6, 24 and 48 hours).

Growth on MacConkey agar

Isolates were introduced into agar plates containing MacConkey agar by use of a sterile wire loop. The agar is selective and differential medium that is designed to isolate and differentiate gram-negative bacteria based on their ability to ferment lactose. Pink colonies on the MacConkey agar confirmed the bacteria to be gram negative.

Salt Tolerance

The nutrient broth was prepared by use of sodium chloride (65 g) (1, 2, 3, 4, and 5%) and dextrose (1.0 g) and allowed to settle at room temperature. Two to three colonies of the isolates were picked and inoculated into the broth and incubated. Turbidity was observed after 12 and 24 hours.

Oxidase production

A filter paper was soaked with the substrate (tetramethyl-p-phenylenediamine dihydrochloride) and moistened with sterile distilled water. The bacterial colony was then picked using a wooden loop and smeared on the filter paper. Appearance of deep-purple colour was observed after 15 seconds.

Catalase Test

Slide method test was used for catalase test. Small amount of bacterial colony was transferred to the surface of a clean, dry glass slide by use of a sterile wire loop and a drop of 3% hydrogen peroxide added

onto the slide and mixed. The rapid elaboration of oxygen bubbles after five seconds confirmed the bacterium catalyzes the breakdown of oxygen from hydrogen peroxide.

Pathogenicity Test

Isolates suspected to be *A. tumefaciens* were inoculated onto an indicator carrot disc bioassay and potato disc bioassay.¹⁰ The same was inoculated onto healthy rose plants and observed.

Carrot Disc Assay

Carrots were obtained from a local market in Rongai. The collected carrots were then sterilized with 1-2% sodium hypochlorite and rinsed in three changes of sterile distilled water. Aseptically, the discs were prepared by chopping into smaller discs using a sterile surgical blade. The discs were placed in sterile petri dishes lined with sterile filter paper. Sterile procedures were followed and one hundred microliters of the inoculum was overlaid on each disc and sealed with parafilm and incubated in growth chamber at 28°C for two weeks with continuous observation for tumor formation from meristematic tissues around the vascular system.

Potato Disc Assay

Potatoes were obtained from local market in Rongai. The collected potatoes were sterilized with 1-2% sodium hypochlorite and rinsed in three changes of sterile distilled water. Aseptically, the discs were prepared by chopping into smaller discs using a sterile surgical blade. The discs were placed in petri dishes containing water agar. According to 10 recommendations, each disc was overlaid with 100µL of the inoculum and sealed with parafilm and incubated in growth chamber at 28°C for two weeks with continuous observation. The discs were then stained with 5% Lugol's Iodine (Iodine 5%, and potassium iodide 10%) for thirty (30) minutes and examined under microscope for tumor formation, where there was tumor formation, the starch was utilized.

Data Analysis

Quantitative data was analyzed using statistical analysis software Genstat Discovery Edition 15 (VSN International Ltd. 2015) while qualitative data was assessed visually and through the microscope. Treatment means for quantitative data were

compared using revised LSD test at the 0.05 level of probability using Fisher's protected LSD.

Results

Effect of Amino Oligosaccharins and Alternaria Fine Protein on Incidence and Severity of Crown Gall

Disease severity was categorized into mild, moderate and severe (Table 1). Rose plants treated with various rates of amino oligosaccharins and alternaria fine protein had mild severity rates compared with the control plots in both sites. The incidence of the disease was assessed fortnightly for ten weeks (Table 2). In Winchester, there was no significant differences in plots applied with amino oligosaccharin and alternaria fine protein at the rate of 1.5g/L and plots applied with copper based fungicide. These plots showed a significant reduction in number of galls after the tenth week. In Bahati however, plots treated with amino oligosaccharin at 1.5g/L showed reduced number of galls on the tenth week after application compared with other treatments. The least incidence was observed in control plots in both farms.

The incidence and severity of the disease was assessed in each plot and control plots had the greatest incidence. Based on the progress of the disease after ten weeks of assessment, the least incidence was reported on plots treated with amino oligosaccharin and alternaria fine protein at 1.5g/L and were not significantly different from the plots treated with copper based fungicides. The severity index shows infection being severe in control plots after ten weeks of assessment.

Effect of Amino Oligosaccharins and Alternaria Fine Protein on the Galling and Size of Galls

There were significant differences in number of galls in each treatment and in the two sites ($P \leq 0.05$) (Table 3). Application of the different rates of amino oligosaccharins had greater effect on the number of galls in Bahati than in Winchester. There was an increase in the number of galls in all the treatments in the second and fourth week after treatment application. However, decline in the number of galls was noticed sixth week after treatment application and it was least in plots treated with amino oligosaccharins at the rate of 1.5g/L. Application of amino oligosaccharins at 1.5g/L had greater effect on

galling formation reducing the number significantly from 2.49 in the fourth week to 1.40 in the eight week and to 1.08 in the tenth week in Winchester while in Bahati, the number reduced significantly from 1.54 to 1.03 ten weeks after treatment application. The

results were not significant different from the plots treated with copper at 2 mL/L in both farms. However, control plots had the number of galls increasing every week in both farms.

Table 1: Severity of Crown Gall Affected by Various Treatments Application

Treatment	Disease Severity		
	Mild	Moderate	Severe
Winchester			
Amino oligosaccharins 0.5g/L	69.0bc	14.8b	14.8cd
Amino oligosaccharins 1g/L	59.1c	19.7a	19.7cd
Amino oligosaccharins 1.5g/L	83.7a	9.9c	4.9d
Copper at 2mL/L	93.6a	4.9d	4.9d
Control	9.9e	14.8b	73.9a
Bahati			
Amino oligosaccharins 0.5g/L	57.1c	19.7a	19.7cd
Amino oligosaccharins 1g/L	64.0bc	9.9c	24.6c
Amino oligosaccharins 1.5g/L	78.8ab	4.9d	14.8cd
Copper at 2mL/L	83.7a	14.8b	4.9d
Control	29.6d	14.8b	54.2b
Mean	63.1	12.8	23.6
LSD (P≤0.05)	18.6	3.8	16.3
CV (%)	25.9	5.3	22.8

Means followed by the same letters along the columns are not significant different at 5% level of probability

Table 2: Disease incidence progress as affected by various treatments application

Treatments	Average number of galls				
	Pre- Treatment	2 weeks	4 weeks	6 weeks	10 weeks
Winchester					
Amino oligosaccharins 0.5g/L	1.4f	1.5e	1.4g	2.1bc	3.3a
Amino oligosaccharins 1g/L	2.1de	1.9d	1.5fg	1.3d	1.2bc
Amino oligosaccharins 1.5g/L	3.4a	1.9d	1.7ef	1.3d	1.1c
Copper at 2mL/L	2.5cd	2.0d	2.0cd	2.0c	1.9b
Control	1.5f	1.5e	1.9de	2.4ab	3.6a
Bahati					
Amino oligosaccharins 0.5g/L	2.0e	2.6ab	2.3ab	2.1bc	1.9b
Amino oligosaccharins 1g/L	2.3de	2.1cd	2.2bc	1.5d	1.0c
Amino oligosaccharins 1.5/L	2.9bc	2.2cd	2.0cd	1.5d	1.0c
Copper at 2mL/L	3.3ab	2.8a	2.0cd	1.6d	1.4bc
Control	2.0e	2.4bc	2.5a	2.5a	3.3a
Mean	2.34	2.09	1.95	1.83	1.97
LSD (P≤0.05)	0.49	0.31	0.24	0.31	0.74
CV (%)	0.4	0.16	0.1	0.17	0.97

Means followed by the same letters along the columns are not significant different at 5% level of probability

Table 3: Effect of amino oligosaccharins at different rates on the number of galls induced by *A. tumefaciens* in Winchester and Bahati

Treatment	Weeks after treatment application				
	2	4	6	8	10
Winchester					
Amino oligosaccharins 0.5g/L	1.99abcd	1.92de	2.05d	2.48b	2.56ab
Amino oligosaccharins 1g/L	2.38ab	2.78ab	2.58bc	2.48b	2.29bc
Amino oligosaccharins 1.5g/L	2.11abc	2.49bc	2.18cd	1.40ef	1.08d
Copper at 2mL/L	2.12abc	2.42bc	2.21cd	1.50def	1.38cd
Control	2.46a	3.12a	3.12a	3.24a	3.30a
Bahati					
Amino oligosaccharins 0.5g/L	1.54de	1.58e	1.54e	2.17bcd	2.67ab
Amino oligosaccharins 1g/L	1.87bcde	2.04cd	2.00d	2.00bcde	2.43ab
Amino oligosaccharins 1.5g/L	1.37e	1.54e	1.46e	1.25f	1.03dcd
Copper at 2mL/L	1.67cde	1.50e	1.46e	1.75cdef	2.56ab
Control	1.92bcd	1.83de	2.79ab	2.42bc	3.17ab
Mean	1.67	1.70	1.85	1.92	2.43
LSD ($P \leq 0.05$) (Trt \times Site)	0.51	0.47	0.44	0.71	1.00
CV (%)	18.00	15.1	15.60	22.30	25.00

Means followed by the same letters along the columns are not significant different at 5% level of probability

Table 4: Effect of amino oligosaccharins at different rates on the size of galls induced by *A. tumefaciens* in Winchester and Bahati

Treatment	Gall size before treatment application (mm)	Gall size 10 wks. after treatment application (mm)	% Reduction in size
Winchester			
Amino oligosaccharins 0.5g/L	10.0	9.3bc	7.0c
Amino oligosaccharins 1g/L	12.2	8.6bcd	29.5abc
Amino oligosaccharins 1.5g/L	14.0	7.6cd	45.7a
Copper at 2mL/L	7.9	6.2d	21.5bc
Control	12.0	16.4a	-36.7d
Bahati			
Amino oligosaccharins 0.5g/L	11.3	10.2b	9.7bc
Amino oligosaccharins 1g/L	13.2	10.3b	22.0bc
Amino oligosaccharins 1.5g/L	12.6	8.7bcd	31.0ab
Copper at 2mL/L	8.9	7.4cd	16.9b
Control	9.3	14.6a	-57.0d
Mean	11.1	9.7	8.9
LSD ($P \leq 0.05$)	1.4	2.5	22.7
CV (%)	4.1	12.4	33.8

Means followed by the same letters along the columns are not significant different at 5% level of probability

There were significant differences in the size of galls before and after treatment applications (Table 4). There was significant reduction in sizes of galls after treatment application with the highest percentage reduction observed in plots treated with amino oligosaccharins at the rate of 1.5g/L and amino oligosaccharins at the rate of 1g/L in both sites. Application of copper fungicide as the standard also resulted in reduced gall sizes in both sites. There was significant increase in gall sizes in control plots.

Biochemical Tests

The biochemical tests for the different isolates of *A. tumefaciens* are presented in Table 5. The gram reaction indicates that the selected isolates were gram negative. The isolates were gram positive for motility, catalase, oxidase, lactose, mannitol, and salt tolerance tests. In terms of shape, the colonies were circular and slightly raised and were cream white in colour with smooth margins (Table 6)

Table 5: Characteristics of the selected strains of *A. tumefaciens*

Biochemical tests	Bahati		Winchester					
	Bht 1	Bht 2	Bht 3	Bht 4	Krn 1	Krn 2	Krn 3	Krn 4
Gram stain	-	-	-	-	-	-	-	-
Motility Test	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
Utilization of Carbohydrates								
Lactose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Salt tolerance	+	+	+	+	+	+	+	+

+: Positive, -: Negative

Bht 1: Bahati sample 1, Krn 1: Karen sample 1

Table 6: Morphological characteristics of *A. tumefaciens*

Character	Nutrient Agar
Shape	Circular, slightly raised
Color	Cream white,
Surface margin	Smooth

Relationship Among Flower Growth Parameters and Crown Galls

From correlation analysis, significant positive correlation was displayed between total number of galls and the size of gall (0.6416, $P \leq 0.05$). However, there was a significant negative correlation between the number of galls and shoot length (-0.695, $P \leq 0.05$).

Discussion

Application of amino oligosaccharins had greater effect on the number of galls; however, the effect depended on the rate of application. There was increase in the number of galls in all the treatments in the second and fourth week after treatment application. However, reduction in the number of galls was noted in the sixth week after treatment application and it was highest in plots treated with

Pathogenicity Test for *Agrobacterium Tumefaciens* Isolates

A. tumefaciens isolates from both farms produced tumors when inoculated on carrot and potato discs. Young galls (tumors) were observed developing at the central part of the carrot and potato discs two weeks after inoculation. However, no symptoms were observed in control discs.

amino oligosaccharins at the rate of 1.5g/L. Amino oligosaccharins and alternaria fine proteins can constitute an important control agent for crown gall in roses. Similar results were reported by.¹¹ In their findings,¹¹ found chitosan and its derivatives to inhibit the growth of *A. tumefaciens* and *Erwinia* spp and displayed highest antibacterial activity against *A. tumefaciens*, *Erwinia* spp with MIC

500 mg/L and 480 mg/L, respectively. Similarly, when oligosaccharin derivatives chitosan were used in controlling *Xanthomonas vesicatoria*, infections were greatly reduced by above 60%¹², reported significant reduction of the disease incidence and delayed symptoms development and the size of the lesions were smaller in comparison with other treatments.

Table 7: Correlation coefficient among flower growth parameters and crown galls

	Shoot length	Size of galls	Stem length	Total galls
Shoot length	-			
Size of galls	0.0193	-		
Stem length	0.4628	-0.1858	-	
Total galls	-0.695*	0.6416*	-0.0809	-

* Significantly correlated

The reduction of number of galls was due to oligosaccharides derivatives unique elicitation of physiological and biochemical changes in roses resulting to induced resistance.¹¹ There is an enhanced activity of enzymes that are linked to defense against plant diseases in response to application of oligosaccharins. Enzymes such as Chi, β -1,3-Glucanase (GLU), peroxidase (PO), phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) are activated and are able to eliminate infectious agents such as *Agrobacterium tumefaciens*.¹² Increase in Phenylalanine ammonia-lyase (PAL) enzyme activity is important as it manages pathogenic infection.

¹Working on tomatoes reported enhanced activities in response to *Ralstonia solanacearum* inoculation in tomato pre-treated with chitosan.

According to^{14,12} oligosaccharins act by inducing several activities of defense enzymes in roses that are involved in pathogen defense. Alternaria fine protein accelerates plant growth vitality, increase proline content, strengthen cellulase and promote the growth of plant cells, strengthen root activity, thereby managing plant diseases. Chitosan, a biopolymer amino polysaccharides has been reported to have strong antibacterial activities against plant pathogenic bacteria such as *A.*

tumefaciens, *Erwinia* spp,¹¹ *Streptomyces scabies*,¹⁵ *Xanthomonas* spp,¹⁶ *Pseudomonas syringae*,¹⁷ However, the activity of chitosan oligosaccharins depends on the concentration^{11,18} and molecular weight¹⁹ and the type of bacteria.²⁰

The mechanism of antimicrobial activity of chitosan and its derivatives remains a mystery as it has not been understood well and it is therefore considered to be complex. However, according to,²¹ at the initial stages chitosan ties with negatively charged components on the bacterial surface by means of electrostatic interactions which changes the permeability of bacterial wall and permits chitosan to get to the internal cell targets subsequently closing down cell division causing death.²²

In this study, bacteria were isolated using nutrient agar media which has been previously used for isolation of *A. tumefaciens* from rose samples with crown gall. A series of biochemical tests such as motility, gram staining, oxidase production and catalase, utilization of lactose and mannitol, salt tolerance (2%) potato and carrot disc assay revealed that the isolated bacterium was gram negative and had the capacity to cause tumor in plant disc sample. The biochemical approaches have been utilized in the past investigations for the recognizable proof of *A. tumefaciens* from crown gall samples from

diverse plant species.¹⁴ Tumor forming ability of the isolates confirmed that they were virulent, however, according to,²⁵ virulence of *A. tumefaciens* was because of the nature of the host, internal physiology of the strains and environmental conditions. Crown gall disease can be severe in rose plantations if young trees are infected. *A. tumefaciens* was found on root surfaces and was effectively isolated from galls. The bacterium was isolated and confirmed using different morphological and biochemical tests.

Conclusion

The study demonstrates the ability of amino oligosaccharins and alternaria fine proteins to be used as an alternative to management of plant diseases. A significant reduction in the number of galls and size following application of amino oligosaccharins and alternaria fine proteins was observed as well as improvement in plant growth. The ability of the treatments to manage the disease can be attributed to enhanced defense enzyme activity. Therefore, this study provides good support

for incorporation of amino oligosaccharins and alternaria fine proteins in the management and yield enhancement of roses.

Acknowledgements

Special acknowledgements go to Higher Education Loans Board (HELB) post graduate scholarship program that awarded me a scholarship that catered for part of my fees, may you never lack so that you continue assisting others in need.

I wish to acknowledge also the contribution of CCIPT Director Mr. Fan Liqiang who provided the samples that were used in this research.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest

The authors do not have any conflict of interest.

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