CHAPTER 6

ANTIOXIDANT ACTIVITY, VITAMIN C CONTENT AND GROWTH OF CHINESE KALE IN RESPONSE TO LEONARDITE (HIGH HUMUS MATERIAL) AND BENEFICIAL MICROORGANISMS

6.1 Introduction

Chinese kale (*Brassica oleracea* var. alboglabra) is a commercial vegetable of northern Thailand and has been for more than two decades. The major areas that produce Chinese kale are located in mountainous areas and production has been increased each year in response to strong demand from domestic and export markets. This has resulted in the use of more agrochemicals such as fertilizers, pesticides, and insecticides which threaten the environment and nearby communities. The use of beneficial microorganisms as microbial inoculants or biofertilizer in preference to agrochemicals in Chinese kale farms is of interest and is one of the government's policies to reduce the use of agrochemicals (Shutsrirung, 2010). Cooper *et al.*, (1998) found that humic acid extracted from soil, peat, or leonardite increased the phosphorus content of turf grass by 3-5%. Humic acid from leonardite significantly increased the root weight of turf grass compared to the control. Studies have shown that humic substances could enhance the germination of maize, wheat, and barley. Root development of maize doubled when humic substances were added to the

nutrient solution (Kononova and Pankova, 1950; Dixit and Kishore, 1967; Chen and Avid, 1990).

Scallbert *et al*. (2005) has commented that the demand for more nutritious foods is increasing, specifically foods that have higher amounts of protein, fiber, and vitamins. Also phytochemicals are an important component of nutritious foods that are known to fight cancer. An example of a phytochemical would be polyphenolic antioxidants that are common in fruits and vegetables. Several studies have found that phytochemicals are present in Chinese kale including glucosinolates, beta-carotene, flavonoids, and vitamin C (Van Poppel *et al*., 1999; Talalay *et al*., 2001; La *et al*., 2009). A study on the antioxidant activities of 22 common vegetables, green tea, and black tea indicated that kale had the highest antioxidant activity against hydroxyl radicals followed by Brussels sprouts, alfalfa sprouts, beets, spinach, and broccoli (Guohua *et al*., 1996). Many studies have shown that crop yield and/or nutritional quality were improved by organic fertilizers and/or beneficial microorganisms. Elazar *et al*., (1989) found that *Azospirillum* could increase the root surface area of maize. Ravi *et al*., (2004) concluded that inoculation of *Azospirillum* strain OAD-2 significantly increased plant height, number of leaves per plant, branches per plant, and total dry mass accumulation in *Gaillardia pulchella* compared to other inoculations and/or uninoculated control. Soltoft *et al*., (2010) concluded that organically grown onions, carrots, and potatoes generally had higher contents of health-promoting secondary metabolites in comparison with the conventionally cultivated ones. Kequan and Liangli (2006) studied the total phenolic contents (TPC)

and antioxidant properties of various vegetables and the results suggested that kale, spinach, broccoli, and rhubarb are good dietary sources of natural antioxidant activities and phenolic compounds.

The aim of this work was to determine the effect of high humus seedling media and beneficial microorganisms on antioxidant activity, vitamin C content, and growth of Chinese kale at seedling stage (20 days after inoculation-DAI) and harvest time (40 DAI).

6.2 Materials and methods

From the previous experiment the two best treatments from the first screenhouse experiment were selected to evaluate the efficiency of high humus seedling media and beneficial microorganisms on antioxidant activity and vitamin C content. The two treatments selected were coconut husk seedling media + 10% leonardite + *Beijerinckia* sp. (VBe 75) and coconut husk seedling media + 15% leonardite + actinomycetes (VAc 77). Selected seedling media (coconut husk compost) was used as control treatment. Randomized complete block design (RCBD) was applied in this experiment (3 treatments with 3 replications). One week after seeding, each of the two selected isolates was applied to the seedlings under treatment. At the seedling stage and harvest time, vitamin C content was measured using high performance liquid chromatography (HPLC) and antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The treatments used in each experiment were as follows:

1) Control (Selected Seedling Media, SSM)

2) SSM+ VBe 75 +10% leonardite

3) SSM+ VAc 77 + 15% leonardite

6.2.1 Pot experiment

6.2.1.1 Preparation of Chinese kale seedlings

The three best treatments from the screenhouse experiment were selected for this study. Plastic seedling trays were used to prepare Chinese kale seedlings. All materials used were analyzed for their chemical properties and humus content using the methods stated aboved. Randomized complete block (RCB) design was applied in this experiment with three replications. Each replication contained 15 to 20 seedlings. Selected seedling media (coconut husk compost) was used as the control treatment. The seedlings were raised under each treatment for 20 – 25 days. Sixseedlings from each treatment were then transferred to pots containing 10 kg of soil.

6.2.1.2 Performance, yield, and nutritional values of Chinese kale 45 to 50 days after transplanting to pots

The soil used in this pot experiment was analyzed for its humus content (humic acid) and chemical properties (pH, N, P, K, Ca, and Mg)(Table 6.1). The methods of analysis were as previously described above. After 45 to 50 days from transplanting, growth performance (shoot and root fresh weight), nutrient content (N, P, K, Ca, and Mg), and vitamin C content were determined.

Table 6.1 Chemical properties of the soil used in the pot experiment

Soil Properties	Analysing Value
pH (H_2O 1:10)	4.87
Organic matter (%)	0.88
E.C. (µS/cm)	53.43
Total N (%)	0.44
Avalaible P (mg/kg)	174.33
Exchangeable-K (mg/kg)	10.67
Exchangeable-Ca (mg/kg)	231.67
Exchangeable-Mg (mg/kg)	21

6.2.2 Analysis of vitamin C content

Fresh Chinese kale was collected for analysis at 20 DAI and at harvest time. Vitamin C content was analyzed as previously described with minor modifications (Hernandez *et al.*,2006, and Yuan *et al.,* 2010). Vitamin C was immediately analyzed on the same day of sample preparation. Chinese kale samples were weighed (1 g) and mixed with 10 ml of the extracting solution (3% metaphosphoric acid (MPA) and 8% acetic acid). The mixture was homogenized with a high-speed blender for 1 min and then centrifuged at 100 rpm at 4°C for 20 min. The supernatant was separated and collected. The extraction procedure was repeated twice on each sample and then the supernatant was again collected and mixed with the previous supernatant. The amount of vitamin C content was determined in the supernatant samples and in the standard (ascorbic acid; 10, 100, 200 µg/ml) by HPLC (Shimadsu, UV-detector). For HPLC analysis, an Ultra Aqueous C18 column (5 particle size, µm 250 mm × 4.6 mm I.D.) was used. The column was flow rate of 1.5 mL/min, the UV detector at 242 nm, where mobile phase A consisted of 100% 10mM potassium phosphate (pH 2.5).

6.2.3 Analysis of antioxidant activity

Chinese kale samples were dried and then extracted by mixing with 10 ml of ethanol using a magnetic stirrer at room temperature for 24 h. Each extracted sample was filtered through whatman No. 1 filter paper. All filtered samples were analyzed with the DPPH method as described by Brand-Williams *et al*., (1995). In brief, 1.8 ml of the DPPH solution (DPPH: Tris buffer: 85% EtOH(1:1:1)) was mixed with the filtered samples or standard (Trolox; 0, 60, 120, 180, 240, 300, 400, 500, 600 µl) to get the final volume of 2.4 ml. Then all samples were placed in the dark for 30 min. The absorbance readings were taken after exactly 30 min at 525 nm by using a UV-visible spectrophotometer (Shimadsu, UV-1601).

6.2.4 SEM (Scanning Electron Microscopy) Study

Representative seedling samples that showed high colonization of each isolate were observed using a JEOL (model JSM-5910 LV) scanning electron microscope (SEM) at EMR Sc CMU (Electron Microscopy Research and Service Center). Small pieces of the sample were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and washed with 0.2 M phosphate buffer pH 7.2. All of the specimens were postfixed in 1% OsO_4 in the same buffer. Dehydration of the fixed specimens was done through a 30, 50, 70, 80, 90, and 100% ethanol series. Dehydrated specimen were immersed in liquid CO_2 in a pressure chamber and were critical point dried in CO_2. The dried specimens were glued to aluminum stubs with silver paint and/or colloidal graphite. Specimens were coated *in vacuo* with 30 nm of gold before micrograph examination with a JEOL SEM.

6.2.5 Identification of beneficial microorganisms by 16S rDNA sequencing

The 16S ribosomal DNA gene was analysed by Thailand Institute of scientific and Technological Research. Sequence data were aligned and compared with available standard sequences of bacterial lineage in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST.

Extraction of bacterial genomic DNA, sequencing, and analysis of homology.

Bacteria were cultivated in 3 ml NA broth. Cultures were shaken at 150 rpm overnight. The bacterial broth was transferred into 1.5 ml eppendorf tubes and then centrifuged at 8000 rpm for 5 min after which the supernatant was removed and discarded. Then 400 µl of lysis buffer and 8 µl of lysozyme (50mg/ml) were added. Samples were vortexed and then incubated at 37 °C for 30 min. Then 4 µl of proteinase K (20mg/ml) and 20 µl of 10% SDS were added. Samples were inverted and then incubated at 37 °C for 30 min. Then 70 µl of 5M NaCl and 55 µl of 10% CTAB were added. Samples were incubated at 65 °C for 10 min. An equal volume of chloroform was added to each sample and mixed. Samples were then centrifuged at 12000 rpm for 5 min. The supernatant was removed and placed in a new eppendorf tube. An equal volume of phenol was added to each sample and mixed. Samples were centrifuged at 12000 rpm 5 for min. The supernatant was again removed and placed in a new eppendorf tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed. Samples were centrifuged at 12000 rpm for 5 min and the supernatant was removed and placed in a new eppendorf tube. An equal volume of

isopropanol was added and mixed. Samples were centrifuged at 8000 rpm for 2 min and the supernatant was discarded. Samples were then washed with 1 ml of 70% ethanol and centrifuged at 8000 rpm for 2 min. The supernatant was removed and discarded. Samples were washed with 1 ml of absolute ethanol and centrifuged at 8000 rpm for 2 min. The supernatant was discarded and cell pellets were allowed to dry. Samples were eluted with 25 µl of D.D.W and stored at -20 °C until use.

Statistical analysis

All data were analyzed by using Statistix8.0 (Tallahassee, FL, USA). Data was analyzed by one-way ANOVA, followed by the least significant difference (LSD) test and significance was accepted at $p<0.05$.

6.3 Results and discussion

6.3.1 Plant growth and nutrient uptake

Shoot and root dry weight of Chinese kale seedlings treated with beneficial microorganisms and leonardite and then transplanted to larger pots and allowed to grow until 40 DAI are presented in Table 6.2. The results demonstrated that treatment 16 (SSM with actinomycetes + 15% leonardite), which was selected from the previous experiment, had a non-significant increase in shoot dry weight (2.41 g/plant) compared to control (1.54 g/plant). Root dry weight was also non-significantly increased in treatment 16 (0.11 g/plant) compared to control (0.08 g/plant). Treatment 12 (SSM with *Beijerinckia* + 10% leonardite), which was selected from the previous experiment, also had non-significant increases in shoot (2.14 g/plant) and root dry weight (0.09 g/plant). Bashan *et al.*, (1990) reported that inoculation of

wheat with *A. brasilense* significantly increased plant dry weight. Diaz-Zorita and Fernandez-Canigia (2009) studied the inoculation of wheat (*Triticum aestivum* L.) seed with a liquid formulation containing *Azospirillum brasilense* INTA Az-39 strain. The crops exhibited more vigorous vegetative growth including a 12.9% greater shoot and 22.0% greater dry matter accumulation.

Other studies also showed similar results. Akbar *et al*., (2009) found that inoculation of *Azospirillum brasilense* (native or Sp7) on *Triticum aestivum* produced significantly higher grain yield by 29% over the control. Additionally, the grains contained more N by 22.8%, more P by 59.5% and more K by 34% when compared to the control plants.

Figure 6.1 Effect of high humus seedling media and beneficial microorganisms on growth of Chinese kale

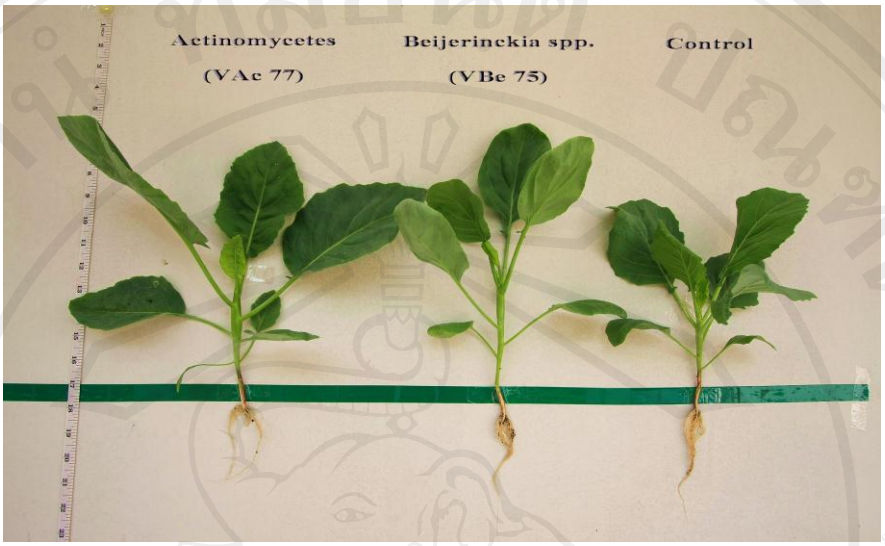

Figure 6.2 Effect of high humus seedling media and beneficial microorganisms on growth of Chinese kale

Table 6.2 Shoot and root dry weight of Chinese kale seedlings treated with beneficial microorganisms and high humus seedling mediagrown until 40 DAI in large pots

Treatments	Dry weight (g/plant)	
	Shoot	Root
1. Control (Selected Seedling Media, SSM)	1.54	0.08
2. SSM + VBe 75 + 10% leonardite	2.14	0.09
3. SSM + VAc 77 + 15% leonardite	2.41	0.11
F – test	ns	ns
C.V (%)	15.20	28.56

ns indicates the effect is not significant at P< 0.05

Table 6.3 Nutrient uptake by Chinese kale seedlings treated with beneficial microorganisms and high humus seedling media grown until 40 DAI in large pots

Treatments	Nutrient uptake (mg/plant)				
	N	P	K	Ca	Mg
1. Control (Selected Seedling Media, SSM)	75.6	6.4	18.5	28.5	8.2
2. SSM + VBe 75 + 10% leonardite	101.1	8.6	44.2	30.3	8.7
3. SSM + VAc 77 + 15% leonardite	113.6	10.3	49.3	34.5	9.9
F – test	ns	ns	ns	ns	ns
C.V (%)	18.39	26.37	34.97	17.02	19.97

ns indicates the effect is not significant at P < 0.05

6.3.2 Vitamin C analysis

Application of beneficial microorganisms together with leonardite showed a tendency toward improved vitamin C content in Chinese kale (Table 6.3). The results indicated that maximum vitamin C content at 20 DAI was obtained by treatment 3 (SSM + actinomycetes (VAc 77) + 15% leonardite) (33.08 µg/ml), followed by treatment 2 (SSM + *Beijerinckia* sp. (VBe 75) + 10% leonardite) (30.07 µg/ml). The control had a vitamin C content of 27.43 µg/ml; however, the difference in the amount of vitamin C at this stage was not significant between the treatments. At 40 DAI the maximum vitamin C content was obtained with treatment 3 with a value of 44.66 µg/ml, which was significantly higher ($p<0.01$) than that of the control (28.39 µg/ml). The vitamin C content of treatment 2 was 30.80 µg/ml and was also higher than that of the control, but was not significant. Other studies have also shown that organic inputs increased the nutritional value of vegetables.

Table 6.4 Effect of high humus seedling media and beneficial microorganisms on vitamin C content of Chinese kale

Treatments	**Vitamin C (µg/ml)**	
	20 DAI	**40 DAI**
1. Control (Selected Seedling Media, SSM)	27.43	28.39 b[/1]
2. SSM + VBe 75 + 10% leonardite	30.07	30.80 ab
3. SSM + VAc 77 + 15% leonardite	33.08	44.66 a
F – test	ns	*
C.V (%)	23.56	32.59

DAI = Days after inoculation

* *indicates the effect is significant at P< 0.05*

[/1] *Values within each column followed by same letter are not significantly different at P < 0.05*

ns indicates the effect is not significant at P< 0.05

6.3.3 Antioxidant activity analysis

The highest antioxidant activity at 20 DAI was obtained by treatment 3 with a value of 1.93 µmol/g while treatment 2 had a value of 1.00 µmol/g and the control value was 0.95 µmol/g (Table 6.4). The antioxidant activity obtained by treatment 3 was significantly ($p<0.01$) higher than that of the other treatments. At 40 DAI antioxidant activity did not differ significantly between the treatments. However, at this stage the maximum antioxidant activity was recorded for treatment 3 with a value of 4.73 µmol/g, followed by treatment 2 with a value of 4.35 µmol/g and compared to the control with a value of 4.34 µmol/g. Our results indicated that antioxidant activity obtained by DPPH assay in treatment 3 was highly significant ($p<0.01$) at 20 DAI when compared with the control. Chinese kale exhibits a high nutritional value because of its high levels of antioxidants and anti-carcinogenic compounds, including vitamin C, carotenoids, phenolic compounds, and glucosinolates (He *et al.*, 2002).

Table 6.5 Effect of high humus seedling media and beneficial microorganisms on antioxidant activity

Treatment	Antioxidant (µmol trolox/g sample)	
	20 DAI	**40 DAI**
1. Control (Selected Seedling Media, SSM)	0.95 b	4.34
2. SSM + VBe 75 + 10% leonardite	1.00 b	4.35
3. SSM + VAc 77 + 15% leonardite	1.93 a	4.73
F – test	**	ns
C.V (%)	31.64	19.95

DAI = Days after inoculation

** *indicates the effect is significant at P< 0.01*

/1 *Values within each column followed by same letter are not significantly different at P < 0.01*

ns *indicates the effect is not significant at P< 0.05*

6.3 Colonization of beneficial microorganisms in Chinese kale root

Chinese kale was planted in sterile seedling media and the roots were examined by SEM. The results showed that actinomycetes (VAc 77) (Fig. 6.4) and *Beijerinckia* spp. (VBe 75) (Fig. 6.5) could live stably in the intercellular space of the Chinese kale roots when compared with Chinese kale roots without BMs (control) (Fig. 6.3).

6.4 Identification of beneficial microorganisms by 16S rDNA sequencing

Beneficial microorganisms were identified by 16S rDNA sequencing. Actinomycetes (VAc 77) was the primary microorganism that was isolated in all experiments. These sequences showed high homology with the sequence of *Streptomyces variabilis* with an overall 99.48% similarity.

Figure 6.3 No colonization in intercellular space of roots of Chinese kale grown in sterile seedling media without inoculation (control)

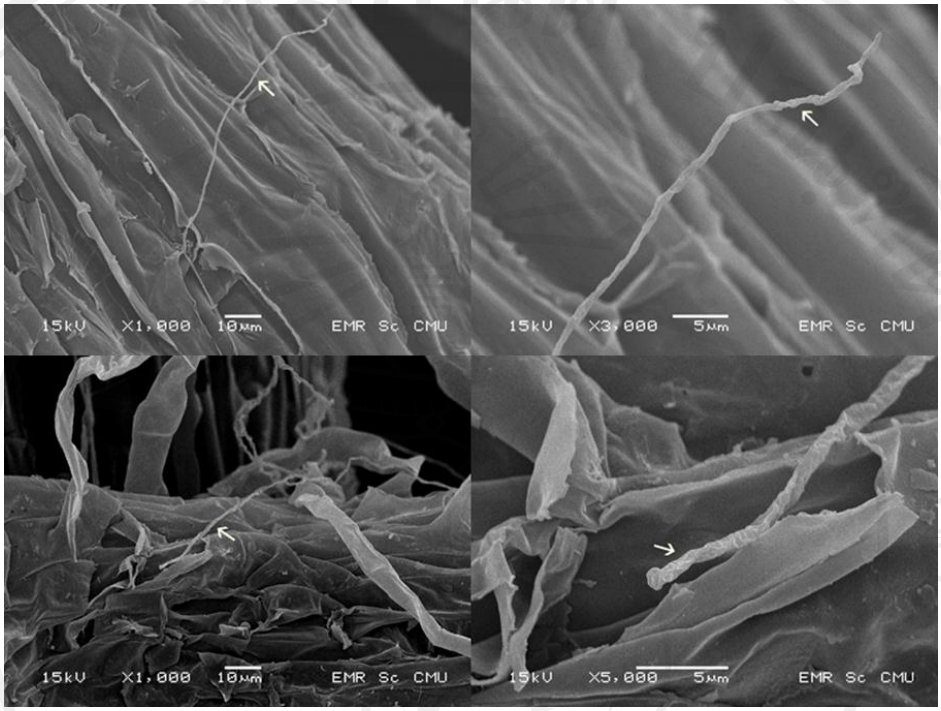

Figure 6.4 Colonization of actinomycetes (VAc 77) in Chinese kale root

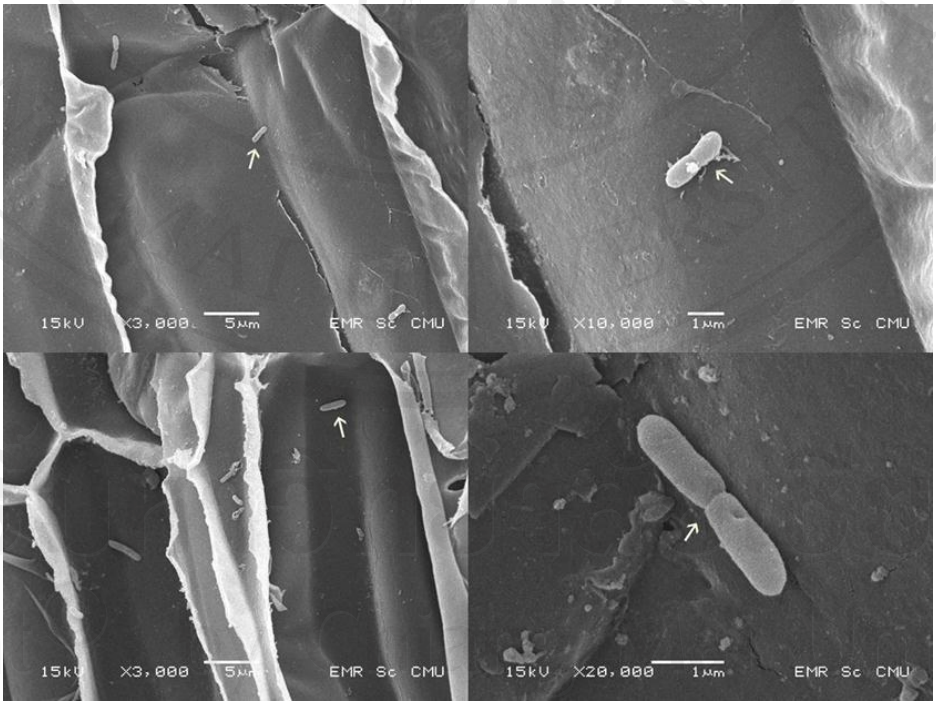

Figure 6.5 Colonization of *Beijerinckia* spp. (VBe 75) in Chinese kale root

6.4 Conclusion

The best mixed media was SSM with actinomycetes plus 15% leonardite, which showed the highest ability to promote good plant growth including the highest shoot and root dry weight. The highest uptake of Ca and Mg in Chinese kale seedlings was also obtained by this treatment. The second best mixed media was SSM with *Beijerinckia* sp. plus 10% leonardite. These two bio-organic mixed media had the best results and could be recommended for using to improve growth parameters and nutrient uptake in Chinese kale seedlings.

Application of high humus materials (leonardite) and beneficial microorganisms in the two mixed media not only improved plant growth and nutrient uptake but also increased antioxidant activity and vitamin C content in Chinese kale. The highest values of vitamin C content and antioxidant activity at 20 DAI were obtained by the two best mixed media and these values were significantly higher than that of the control. This indicated that when selected seedling media (SSM) was mixed with actinomycetes (VAc 77) plus 15% leonardite, the mixed media was able to promote the vitamin C content and antioxidant acitvity. Our results suggested that high humus materials and beneficial microorganisms can assist in ensuring good performance and high quality vegetables after transplanting.