Study on Mitigation of Allelopathy and Autotoxicity in Replanting Problem of Asparagus (*Asparagus officinalis* L.)

アスパラガスの連作障害におけるアレロパシーおよび自家中毒の軽減に関する研究

Rumana Yeasmin

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CHAPTER I

GENERAL INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a hardy perennial vegetable native to the seacoasts of Europe and eastern Asia, where it has been cultivated for over 2,000 years. Early settlers brought asparagus to North America, where it has been grown in home gardens since colonial times. Commercial asparagus production began in this country in the middle of the nineteenth century. The top asparagus importers were the United States (92,405 tones), followed by the European Union (external trade) (18,565 tones), and Japan (17,148 tones) (according to Global Trade Atlas and U.S. Census Bureau statistics, 2004). The United States' production for 2005 was on 218.5 square kilometers (54,000 acres) and yielded 90,200 tones (National Agricultural Statistics Service, 2006) making it the world's third largest producer, after China (5,906,000 tones) and Peru (206,030 tones) (FAOSTAT, 2007). As of 2007, Peru is the world's leading asparagus exporter, followed by China and Mexico. It has been a major cash crop in this cold district for a long time. White asparagus production rapidly changed into green asparagus farming. Green asparagus exceeded white in production yield in 1978, and production has been expanded until today. This vegetable is one of the rapidly exploited cash crops as a conversion caused by rice planting regulation in rice field area or decline of sericulture in mountain areas. In 1998, twenty-four thousand tons of total green and white asparagus were produced in 6,500 ha all over Japan. Major cropping types are combinations of the cropping style (greenhouse forcing planting, greenhouse semi-forcing and open field). The major production areas in Japan are distributed in cold areas (Nagano, Hokkaido, Fukushima Prefectures) and warm areas (Saga, Kagawa, and Nagasaki Prefectures). Production areas of green asparagus have mainly converted from white asparagus in Hokkaido, from rice in Fukushima, from mulberry for sericulture in the Nagano Highland

and as one of the greenhouse crops of warm areas in Nagasaki and Saga. Therefore, the former three and latter two areas are usually categorized into land-utilizing cultivation and intensive cultivation. Once, green asparagus was introduced into the open field all over the country, but stem blight caused non-economic production conditions in the lower, warm areas. Spotted distribution of the production areas, the various cropping styles and the economical position of the crops among growers caused widespread technological gaps. For example, the yield in Nagano was 3.49 t / ha on the open field in 1997 compared with 2.18 t / ha in Hokkaido (Ministry of Agriculture Forestry and Fisheries, Monthly Statistics of Agriculture Forestry and Fisheries, 2010). Asparagus is the most important income crop in Nagano; however, this is only one of the economic crops such as potato, wheat and beet in Hokkaido. Therefore, farmers cannot devote enough time to asparagus cultivation in economic returns exists in the low level areas. The use of plastic film covers contributes to stable yields in Hokkaido. The farmer's motivation is dropping because of low yield and vicious, downward spiral for because it protects against diseases and prolongs the harvest period in Nagano. In Saga, spear yields reached 19.3 t / ha in greenhouses, there are only greenhouse productions in this district. This yield is approximately nine times the yield in open field in Hokkaido. According to ministry of agriculture, forestry and fisheries of Japan in 2010, asparagus production area in Japan showed in **Fig. 1**. The development of the continuous harvesting cultivation system was a breakthrough. Spear growth is supplied from not only rhizome and storage root but also assimilated products from four or five fern in this method. They can continue to harvest through three seasons. Special measures are also taken against as above in this area. Now it is widely cultivated as one of vegetable crop. Asparagus has been used from early times as a vegetable and medicine, owing to its delicate flavor and diuretic properties. The second century physician Galen described asparagus as "cleansing and healing."

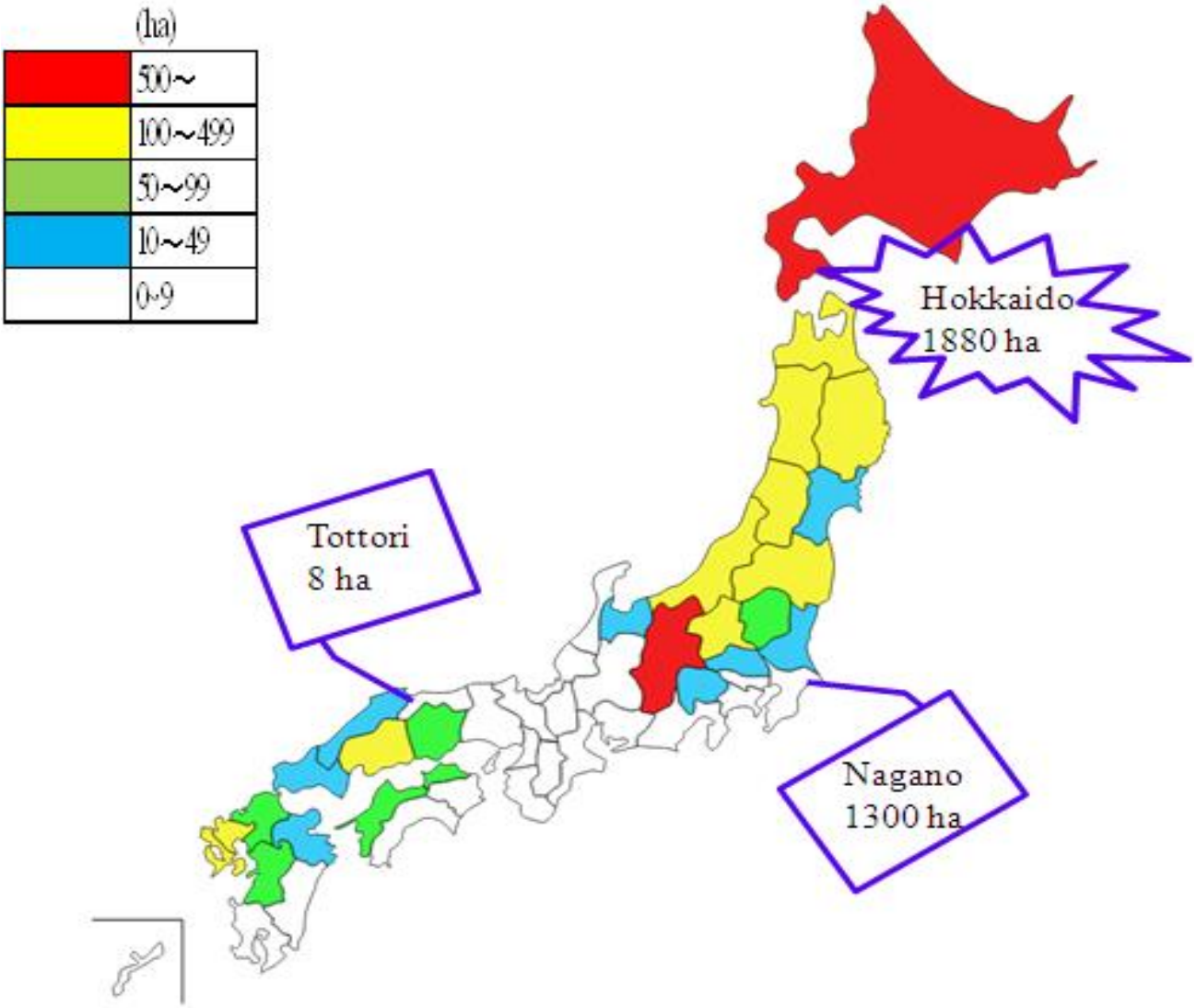


Fig. 1 Asparagus production area in Japan

Ref. Ministry of Agriculture, Forestry and Fisheries of Japan in 2010

Nutrition studies have shown asparagus provides essential nutrients: six spears contain some 135 micrograms (μg) of folate, almost half the adult RDI (recommended daily intake), 545 μg of beta carotene, and 20 milligrams of potassium," notes an article in Reader's Digest, August, 2011. It is also a good source of vitamin B_6, calcium, magnesium and zinc, and a very good source of dietary fiber, protein, vitamin A, vitamin C, vitamin E and K, thiamin, riboflavin, rutin, niacin, folic acid, iron, phosphorus, potassium, copper, manganese and selenium.

Asparagus has shown a marked decline in productivity after many years of continuous harvesting. Grogan and Kimble (1959) found that asparagus production on former asparagus land is less profitable than that on flesh land. Infact, first asparagus establishment generally is not affected, but after a few years of normal yields, both growth and yield are declined by the replanting problem. **Fig. 2 (a)** and **(b)** showed the asparagus field with 6 and 15 years old root residues. With the increasing of root residue asparagus growth decreased. The symptoms of this phenomenon are similar to those described for asparagus early decline. In almost all reports on replant problems, the phenomenon could be related to an early decline of the preceding asparagus crop. In addition, the economic life of asparagus in replanted fields averages only half that of plants planted in new land (Blok and Bollen, 1993). Shafer and Garrison (1986) found that since asparagus possesses large storage roots that are continually dying as the crown grows, large amounts of live, senescing, and dead asparagus root material could be present in fields at any one time which could be responsible for replanting problem. **Fig. 3 (a)** and **(b)** showed the 6 and 15 years old asparagus root residues. These roots and residues apparently release numerous toxic chemical compounds as “allelochemicals” that can inhibit seed germination and seedling, growth of different species as well as asparagus growth, peroxides activity of asparagus seedling and respiration of the crown (Hartung and Stephens et al., 1983).

Fig. 2 (a) Asparagus field with 6 years old root residues

Fig. 2 (b) Asparagus field with 15 years old root residues

Allelochemicals released from the roots and decomposing roots, unbalanced nutrient supply, cold and drought stress and nematodes (Schofield, 1991). Blok and Bollen (1993) conducted an experiment to find out the effect of replanting problem on asparagus, at two locations, each with fields where asparagus production was terminated 1 and 10 years before, biomass of root residues was 4180 and 11060 kg dw ha^{-1} after 1 year and 420 and 1140 kg dw ha^{-1} after 10 years.

Asparagus yield have been much decreased caused by replanting due to allelopathic and autotoxic potentiality (Putnam, 1985). The term "Allelopathy" means an interaction between plants, plant and insects, plant and microorganisms, and plant and animals through bioactive natural chemicals. Allelopathy is already a time-honored concept, starting from the definition of Dr. Hans Molisch in 1937. Allelopathy is an action of natural bioactive chemicals produced by plants to other life. Recently, allelopathy is getting more and more important. Because, this concept helps in the organic or natural farming without or less use of synthetic agrochemicals (herbicide, insecticide, fungicides, etc.). In the past, the meaning of these chemicals in plants seemed to be a pool of energy or reducing agents, or simple wastes. But recently, the allelopathy hypothesis describes the real meaning of these "secondary metabolites" as a tool of immobile plants to protect them from surrounding plants or other life that might attack them, or a tool to communicate each other or to communicate with other life for their survival (Inderjit, 2001). Autotoxicities are considered as special cases of allelopathy, although the same compounds may exert interspecies effects. Autotoxicity is defined as a form of allelopathy in which a species inhibits growth of members of that same species through the production of chemicals that are released into the environment (Blok and Bollen, 1993). This mechanism will result in reduced competition between members of the same species. In cultivation, autotoxicity can make it difficult or impossible to grow the same

Fig. 3 (a) 15 years old asparagus root residues

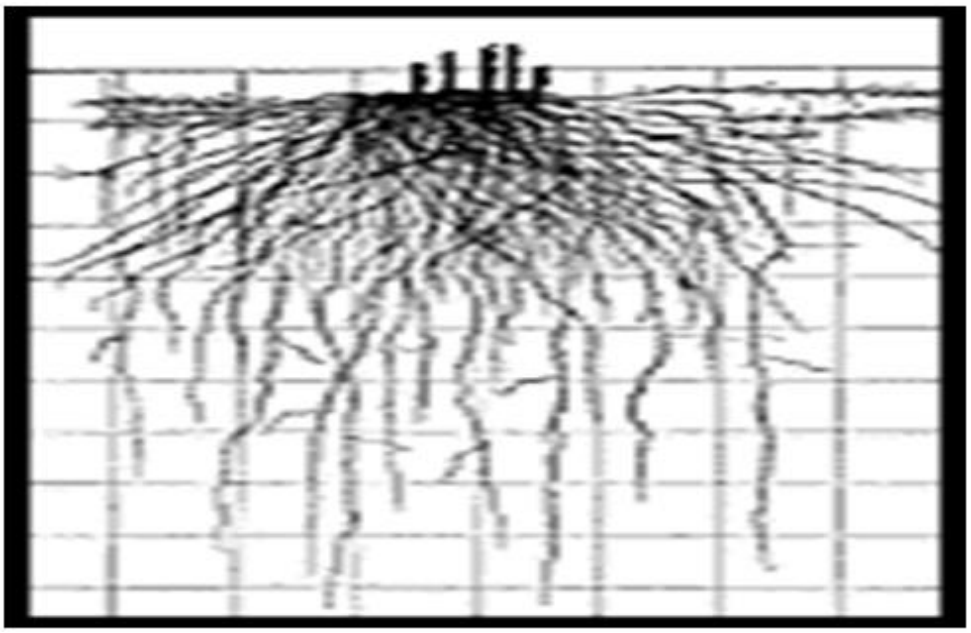


Fig. 3 (b) 6 years old asparagus root residues

species after harvest of a crop. Asparagus stems, root and old litter extracts can inhibit asparagus seed germination (Young and Chou, 1985). These inhibitory compounds were identified as phenolic acids and saponins (Lake et al., 1993). Vokou et al. (2003) reported that any allelopathic interaction involves at least one allelochemical. Such chemicals are liberated from live or decaying plant biomass through volatilization, leaching, root exudation or decomposition of plant residues, further affecting positively or negatively microbes and the processes in which they are involved. In general, roots appear to be a more important source than above-ground parts. Within the soil environment the free movement of allelochemicals, is now known to influence a wide variety of soil related and crop management processes, including soil fertility, nitrogen transfer, weed control, crop compatibility within rotations, residue management, plant growth, and the development and the maintenance of disease suppressive soils (Einhellig, 2002). It has been commonly assumed that there are more than 500,000 plant species and more than 30,000 secondary natural chemicals in this world. However, we are sure that there are still many natural chemicals unidentified to us.

Recently, AC has been used to test for allelopathic effects on plant growth; however, use of AC to test for allelopathy may lead to erroneous results if one does not control for confounding effects of AC on both test and focal species growth. Most studies using AC in allelopathy experiments do test for effects of AC on the growth of test species in the absence of the

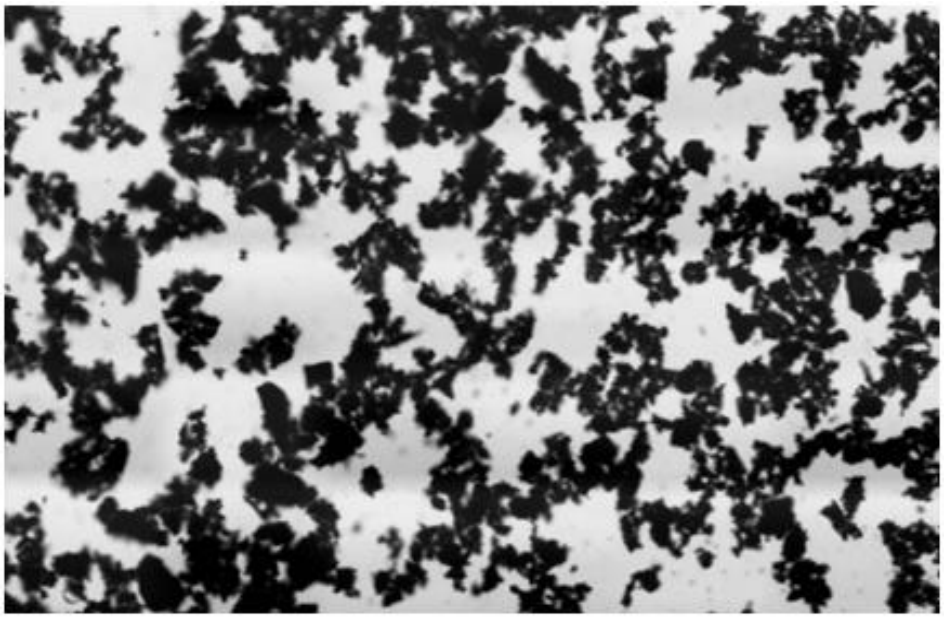


Fig. 4 (a) A micrograph of palm activated charcoal under bright field illumination on a light microscope. Ref. Laine et al., 1989

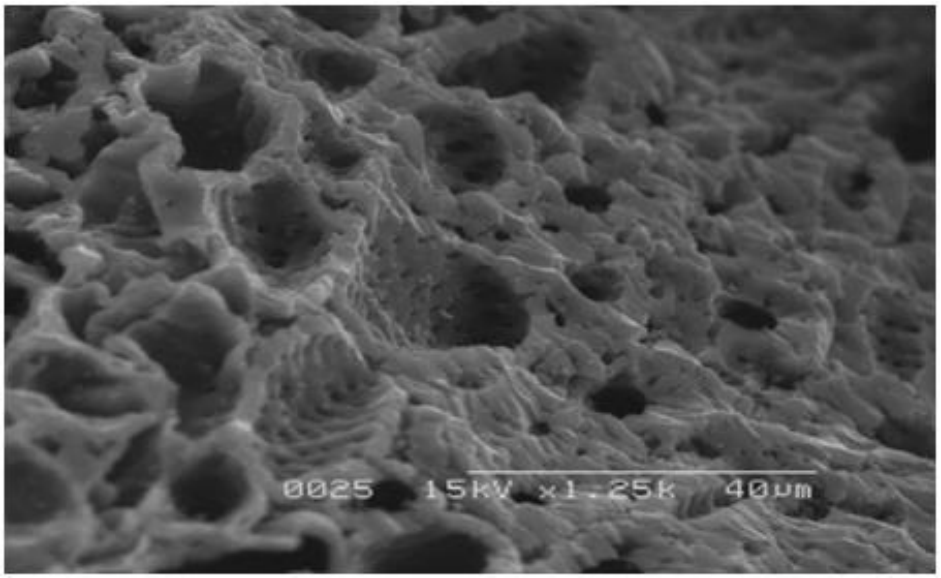


Fig. 4 (b) Palm activated carbon, as viewed by an electron microscope. Ref. Nishihara et al., 2005

potentially allelopathic agent to control for unwanted growth effects of AC that accompany its intended use as a minimizer of allelopathic effects. AC affects the growth of several plant species, likely via its effects on potting medium properties, especially nutrient availability (Lau et al., 2007). Inderjit and Callaway (2003) recommend fertilizing pots to minimize the effects of trace concentrations of nutrients contributed by AC. A variety of studies have used activated carbon (AC) to neutralize the effects of allelochemicals (Mahall and Callaway, 1992; Nilsson, 1994; Callaway and Ashehoug, 2000; Inderjit and Callaway, 2003; Kulmatiski and Beard, 2006). **Fig. 4 (a) and (b)** showed the micrograph of palm activated charcoal under bright field illumination on a light and electron microscope, respectively. AC, with a large surface area and pore volume, as well as its polarity, has a tremendous adsorptive capacity and complex chemical and physical properties. The activity can be separated into adsorption, mechanical filtration, ion exchange, and surface oxidation (Cheremisinoff and Morresi, 1978).

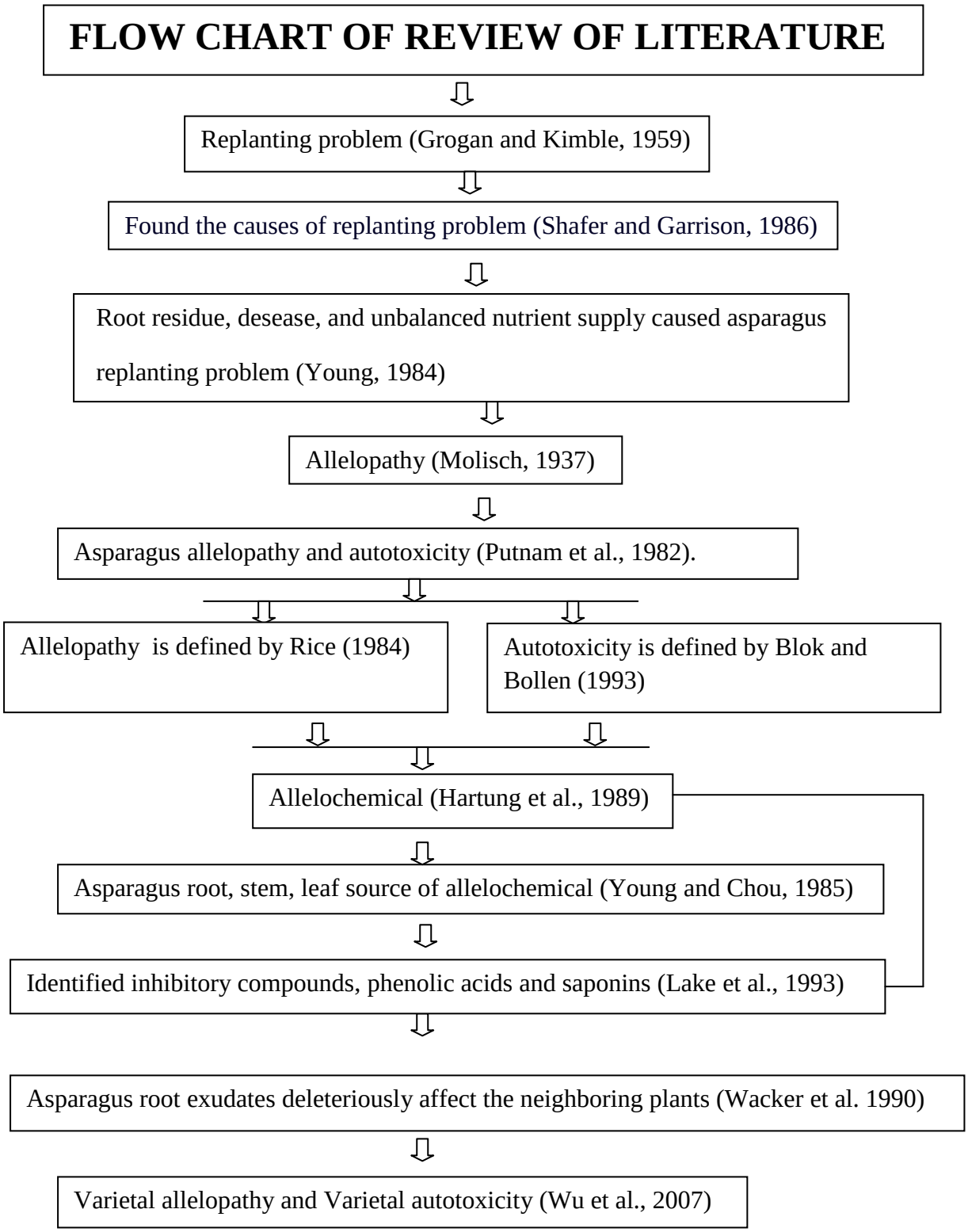
Overall Scope and Study Objectives:

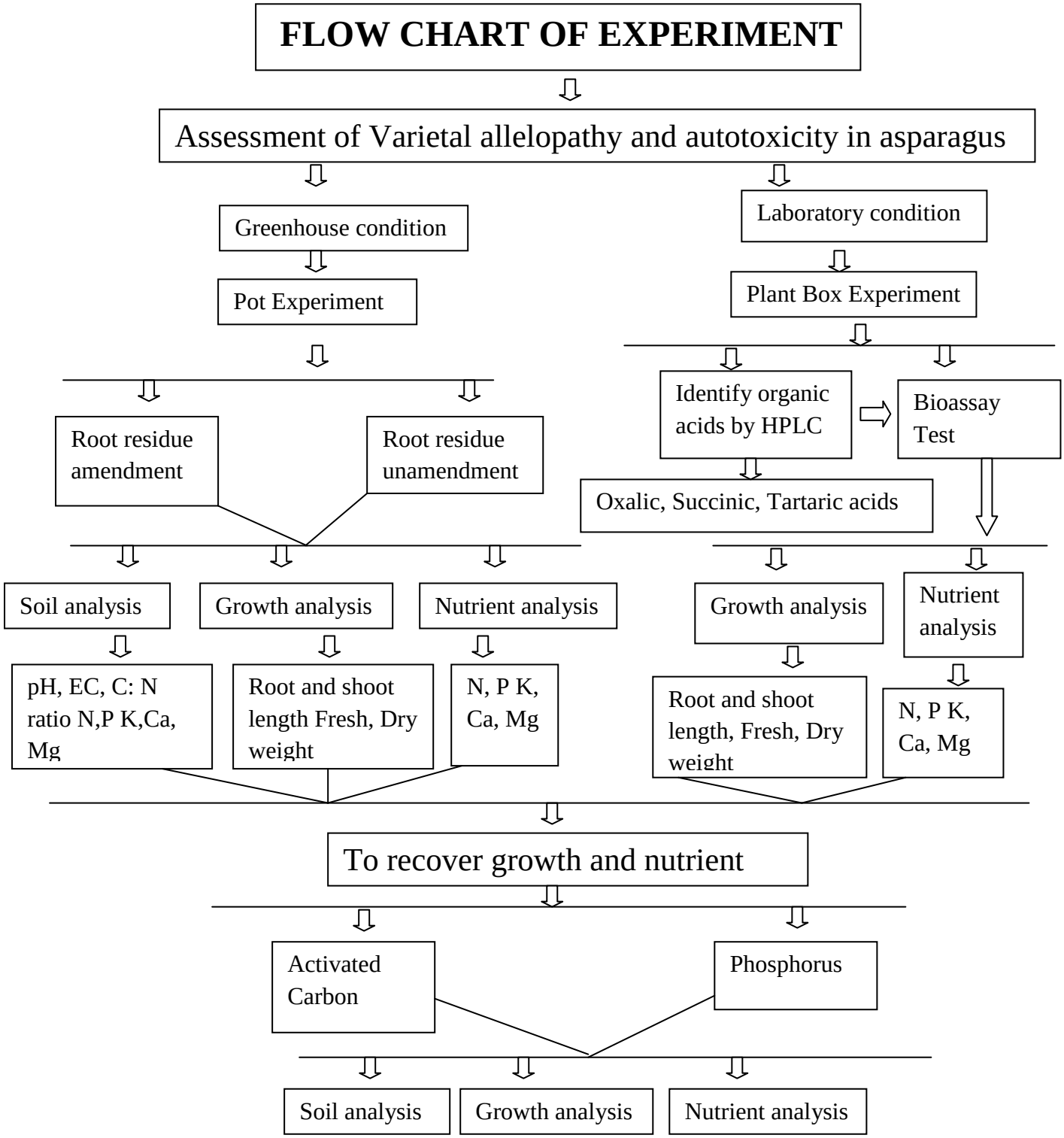
1) To identify the variety of asparagus which is more resistant to allelopathy and autotoxicity under replanting conditions.

2) To investigate the mechanism of allelopathy and autotoxicity in aparagus which is exudated from the roots of asparagus.

3) Replanting is a serious problem in crop production, therefore to be able to mitigate the problem by using activated carbon and phosphorus.

FLOW CHART OF REVIEW OF LITERATURE




FLOW CHART OF EXPERIMENT
Assessment of Varietal allelopathy and autotoxicity in asparagus
Greenhouse condition
Pot Experiment
Laboratory condition
Plant Box Experiment
Root residue amendment
Root residue unamendment
Identify organic acids by HPLC
Bioassay Test
Oxalic, Succinic, Tartaric acids
Soil analysis
Growth analysis
Nutrient analysis
Growth analysis
Nutrient analysis
pH, EC, C: N ratio N,P K,Ca, Mg
Root and shoot length Fresh, Dry weight
N, P K, Ca, Mg
Root and shoot length, Fresh, Dry weight
N, P K, Ca, Mg
To recover growth and nutrient
Activated Carbon
Phosphorus
Soil analysis
Growth analysis
Nutrient analysis

CHAPTER II

Root Residue Amendment on Varietal Allelopathy and Autotoxicity of Replanted Asparagus (*Asparagus officinalis* L.)

1. Introduction

Root residue exudates of asparagus inhibited the roots and shoots growth of asparagus own seedlings, suggesting autotoxicity was a possible mechanism for the problem of declined yield and replanting problem (Young, 1984). Root residues entering into the soil can affect growth and yield through changes in microbial activities and N immobilization or availability (Hungate et al., 1997). Batish et al. (2009) reported that phenolics were present in root residue amended soils, and growth reduction in root residue amended soils was concomitant with the amount of phenolic compounds. Root residues can lead to increased N immobilization in cropping systems by increasing the amount of carbon relative to nitrogen (Lam et al., 2012). Crop residues added to soils have been shown to reduce the recovery of fertilizer (Kongchum et al., 2007). However, other potential causes for the replanting problems, include allelopathy and autotoxicity. Allelopathy refers to the effects of one plant on another caused by the release of inhibitory substances into the environment through root exudation, followed by leaching and volatilization, or through the decomposition of plant residues (Batish et al., 2007). Autotoxicity is a form of intraspecific allelopathy that occurs when a species releases chemical substances that inhibit or delay the germination and growth of plants of the same species (Putnam, 1985). Additionally, the chemical interactions between varieties within the same crop species can be classified as "varietal allelopathy" and "varietal autotoxicity" (Wu et al., 2007). Varietal allelopathy occurs when plants of a given variety release chemical substances that inhibit or delay germination and growth of other varieties of the same crop species. On the other hand, varietal autotoxicity occurs when plants of a given

variety release chemical substances that inhibit or delay germination and growth of the same variety. The chemicals involved in allelopathic interactions are present in all plant parts such as leaves, roots, stems, inflorescence and even pollen grains (Rice 1984). Among these modes, the role of roots is significantly more as these are in direct contact with soil and contribute allolechemicals into the growth medium. Rather, root exudates play an important role in plant-plant interactions and deleteriously affect the neighboring plants (Bais et al., 2006). Many researchers have confirmed the allelopathic activity of chemical substances released by asparagus (Hazebroek et al., 1989; Lake et al., 1993; Peirce and Miller, 1993; Shafer and Garrison, 1986; Yang, 1982; Young, 1984; Young and Chou, 1985) and allelochemicals are considered to be one of the potentially important causes of the asparagus replanting problem (Putnam, 1985).

Allelopathy and autotoxicity are related to environmental stresses such as nutrient deficiencies. The allelochemicals change the concentration of soil nutrients. In addition, the concentration of soil nutrients also influences the concentration of allelochemicals in the plant. The avoidance of allelopathic effects between plants, or the exploitation of beneficial interactions in a rotation or a mixed cropping system, may have direct bearing on crop yield (Rizvi and Rizvi, 1992). Improved yields associated with crop rotation are often attributed to reduced disease incidence, and believed to be due the successful management of natural communities (Cook, 1993). Moreover, little is known about varietal allelopathic and autotoxic interaction in terms of continuous replanting of asparagus in soil amended by root residues. Therefore, the purpose of this investigation was to evaluate asparagus varietal resistance to allelopathy and autotoxicity, with a bias in the adequate rotational patterns that would be beneficial in mitigating the replanting problem caused by root residues amended soil.

2. Materials and Methods

2.1 Planting Materials

Two asparagus seed varieties; UC157 (called as Welcome in Japan) and Gijnlim of USA and European origin, respectively were procured from a local commercial seed company in Japan.

2.2 Characteristics of the Sandy Soil

Physico-chemical properties of the soil are illustrated in **Table 1**. Sandy soil used in this experiment was first sterilized at 121°C for 15 min in an automatic high pressure steam sterilized autoclave (MLS-2420; Sanyo, Tokyo, Japan). Prior to showing, the soil was characterized for EC and pH of the soil: water suspension (1:5 w/v). EC and pH were measured with EC and pH meters (Horiba DS-14 and Accumut M-10, TOA electronics Ltd., Tokyo, Japan, respectively). Exchangeable cations (K^+, Ca^{2+}, $Mg^{2+,}$ and Na^+) were measured using an atomic absorption spectrophotometer (Model Z-2300; Hitachi Co., Tokyo, Japan) after extraction with neutral ammonium acetate.

2.3 Assessment of Varietal Resistance to Allelopathy and Autotoxicity

Fresh pre-germinated 20 days old seedlings of UC157 and Gijnlim were separately sown in sandy soil- filled pots (790 cm^3), and left to grow for 50 days in the greenhouse conditions at the Field Science Center, Tottori University, Tottori City, Japan **(Fig. 5)**. The greenhouse conditions were 20°C to 25 °C, 10 h to 12 h light and 10 h to 12 h dark, 70 to 80% relative humidity and

Table 1 Soil physico-chemical properties

Soil Parameter	Value
EC (1:5) water	0.03 ds· m^{-1}
pH	6.36
Total N	0.02%
Available-P	1.5 mg P2O2 100 g^{-1}
Exchangeable K^{+}	0.06 cmol·kg^{-1}
Exchangeable Ca^{2+}	0.34 cmol·kg^{-1}
Exchangeable Mg^{2+}	0.45 cmol·kg^{-1}
Exchangeable Na^{+}	0.10 cmol·kg^{-1}
CEC Cation exchange capacity	2.40 cmol·kg^{-1}
Bulk density	1.47 g·cm^{-1}
Infiltration rate	30 mm·min^{-1}
Hydraulic conductivity	0.05 cm·sec^{-1}
Texture	Sand

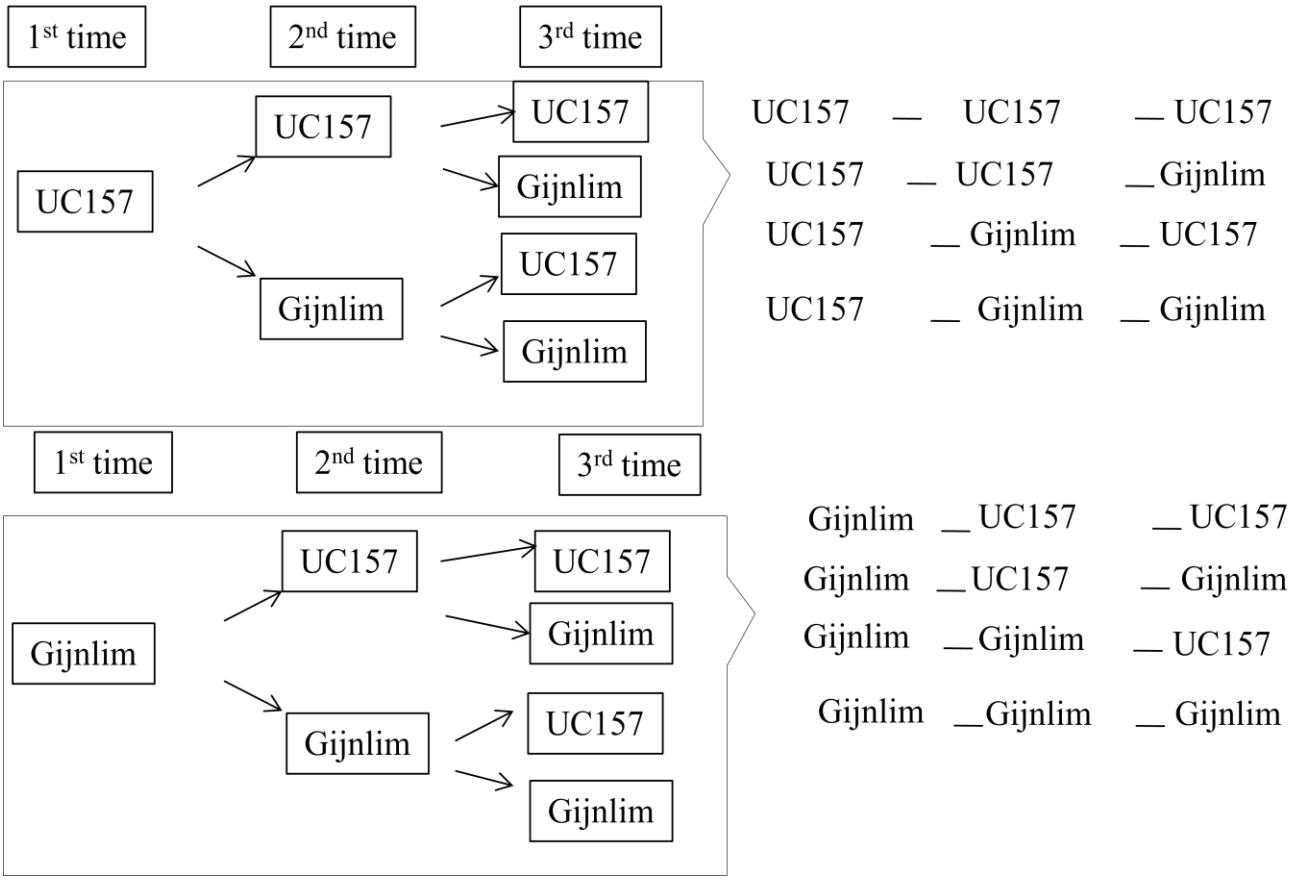
Fig. 5 Asparagus replanting under greenhouse condition

150-200 µmol· m^{-2} ·s^{-1} light intensity. First planting of each variety without root residue amendment served as the control. After 50 days, seedlings of both Gijnlim and UC157 were harvested, their roots cut off, washed thoroughly, air-dried and incorporated back into the same pot soil at 2 g·kg^{-1} soil. Again, pre-germinated 20 days old individual seedlings of both UC157 and Gijnlim were replanted in the root residue-soil mixture for the second (first replanting) planting for another 50 days. The third (second replanting) planting also followed the same procedure as above. Prior to the third plantings, additional fresh root residues (at the same rate) were incorporated into each pot. During all cultivation cycles and treatments, irrigation was done at 50 ml·day^{-1} per pot; fertilization immediately after each planting, and then at 15 day intervals with 100 ml of a solution containing 10 mg·L^{-1} of N (NH_4Cl 1.5 mg·L^{-1} + 8.2 mg·L^{-1} HNO_3), 8 mg·L^{-1} P, 27 mg·L^{-1} K, 4.0 mg·L^{-1} Ca, 0.1 mg·L^{-1} Mn and 0.1 mg·L^{-1} B. All treatments were replicated three times. The inhibition (%) was calculated using the equation:

$$\text{Inhibition (\%)} = (1 - Xt/Xc) \times 100 \qquad (1)$$

In this equation, Xc denotes the length or dry weight of the roots or shoots of the control and Xt represents the corresponding length or dry weight in the second (first replanting) and third (second replanting) planting. The above equation was used to compare the performance of the two varieties UC157 (U) and Gijnlim (G) in the following rotational combination patterns according to **Layout 1** for varietal allelopathy: UG, GU (first replanting) and UGU, UUG, UGG, GUG, GGU, GUU (second replanting) and autotoxicity: UU, GG (first replanting) UUU, GGG (second replanting), respectively.

2.4 Plant Growth and Nutrient Uptake Measurements



Layout 1: Rotational cultivation system

After harvesting at the end of each planting, all seedlings were carefully separated into roots and shoots, thoroughly cleaned, blotted dry between absorbing paper and their lengths were measured. Root and shoot dry weights were measured after oven drying at 70°C for 72h. To determine nutrient uptake and the growth inhibition by the root residues following procedures were performed. All dry roots and shoots were combined and ground to a fine powder using a stainless ball mill, and analyzed for total nutrients N, P, K, Ca and Mg using standard procedures. Total N content was determined by the dry combustion method with an automated C-N coder (Model MT 700; Yonaco, Tokyo, Japan). Total major mineral nutrients K, Ca and Mg were determined after digestion with a H_2O_2-H_2SO_4 mixture. Total P in the digested mixture was determined colorimetrically with a spectrophotometer (Model U-2001, Hitachi Co., Tokyo, Japan) using the phosphomolybdate blue method (Murphy and Riley, 1962). The total amounts of K, Ca, and Mg were determined with an atomic absorption spectrophotometer (Model Z-8100, polarized Zeeman; Hitachi Co., Tokyo, Japan). Inhibition, expressed as a percentage, for nutrient uptake was calculated by using equation 1.

2.5 Chemical Analysis of Soil after Subsequent Replanting

To check whether root-residue incorporated into the soil alters soil nutrient status; *Asparagus officinalis* root residue amended soils were analyzed for pH, electrical conductivity (EC), C: N ratio, and total N, P, K, Ca and Mg. The pH and EC of soil were measured in 1: 10 soil–water extracts by using the pH and EC meters (HM-30S and CM - 30R-DKK, TOA electronics Ltd., Tokyo, Japan), respectively. Total mineral nutrients N, P, K, Ca and Mg were measured as described above.

2.6 Statistical Analysis

Experimental data presented are the means of three replicates. Statistical analyses were executed using Stat view software. One-Way analysis of variance (ANOVA) was used to compare the percentage of inhibition in growth and nutrient uptake by Tukey's protected multiple- comparison test at $P<0.05$. The percentage data was transformed according to Lam et al. (2012) to conduct the appropriate tests for normality and equality of variance before proceeding with the analysis. Data is presented as mean ± SE of the three replications. Principle component analysis (PCA) was performed using XLSTAT 2011 to clarify total data variability with respect to co-relationships between growth, nutrient uptake, and soil physicochemical characteristics after the first and second replanting. Moreover, each rotational combination was treated as categorical data, converted to dummy variables and PCA performed along with other variables. Due to very low cumulative contribution ratio, some dummy variables were omitted.

3. Results & discussion

3.1 Varietal Resistance to Allelopathy and Autotoxicity

The potential of allelopathy and autotoxicity on root and shoot growth pattern between two asparagus varieties with different rotational combinations are illustrated in **Table 2**. Asparagus root residues significantly ($P \leq 0.05$) affected root and shoot growth in both varieties under different rotational combinations when compared to the unamended (control) soil; with the second replanting showing a higher inhibition than the first. However, the negative effect was more on root than shoot. In allelopathic treatments after the first replanting, the highest and lowest inhibition of root (26 and 10 %) and shoot (21 and 8%) growth occurred in the UG and

Table 2 Growth inhibition of two asparagus varieties with different rotational combinations (RC) after the first and second replantings

			Percent inhibition			
			Length		Dry Weight	
PT	Treatment		Root	Shoot	Root	Shoot
First RP	Autotoxicity	UU	49±2.1a	45±1.8a	48±4.6a	46±2.6a
		GG	29±2.6b	25±1.5b	29±7.3b	26±4.9b
	Allelopathy	UG	18±0.6b	14±1.6b	26±2.1b	21±1.4b
		GU	10±1.3c	8±0.8c	17±1.5c	14±4.2c
Second RP	Autotoxicity	UUU	83±0.3a	72±3.6a	91±0.7a	78±1.6a
		GGG	53±2.1b	49±1.4b	54±0.9b	52±1.9b
	Allelopathy	UUG	49±1.0c	46±1.2c	52±0.6c	47±1.1c
		GUU	45±2.1c	37±1.8c	49±0.6c	41±0.7c
		UGG	43±0.3c	36±8.3c	43±0.7c	38±1.0c
		GGU	38±0.3c	32±5.3c	38±1.8c	35±1.6c
		UGU	34±0.8c	29±6.2c	36±2.5c	31±0.6c
		GUG	31±4.2d	24±1.4d	31±1.3d	28±1.9d

Note: Data presented as mean ± SE, (n = 3). Lettering in the table refers to comparisons for the first and second replanting (Tukey's protected multiple-comparison test, $P \leq 0.05$). Inhibition percent was calculated by using equation 1. U, UC157; G, Gijnlim; the order of the letters represents the two varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Listed length and weight suggests the values are actual lengths (cm) and weights (g). Here, PT denotes to planting time.

GU rotations, respectively. After the second replanting, the highest and lowest inhibitions were found in the UUG and GUG rotations, respectively.

Growth inhibition was significantly ($P \leq 0.05$) higher in UC157 than in Gijnlim in the first and second replanting in the same rotation with each of variety **(Table 2)**. For example, in autotoxic treatments, both the UU (49%) and UUU (91%) rotations showed significant ($P \leq 0.05$) higher growth inhibition than the GG (29 %) and GGG (54%) rotations. This inhibited growth is likely caused by varietal and rotational differences in the susceptibility to the type and total quantity of allelochemical constituents. Inhibitory effects of allelochemicals varied with varietal differences (Chung et al., 1997). Exudates from root residues are one of the major sources of potential allelochemicals to the soil rhizosphere (Rice, 1984, Bertin et al., 2003). They contain a variety of chemical compounds including amino acids, organic acids, sugars, phenolic acids, and other secondary metabolites, and serve as an important medium of root-based interactions with other microorganisms (bacteria, actinimycetes, pathogens, fungi, and insects) in the soil (Walker et al., 2003). In this study, root and shoot growth inhibition ranged from 10 to 91% and 8 to 78 %, respectively in the same combination or the different combinations with each of variety. Lam et al. (2012) reported that root residue amendment treatments were decreased root and shoot biomass by 16 and 19 % respectively. Therefore, root residues could have released some allelochemicals into the soil through exudation or degradation of the root residue and these might be interfering the growth of asparagus. Increased autotoxication in asparagus root residues due to accumulation of allelochemicals from subsequent replanting could have been responsible for growth inhibition (Young and Chou, 1985).

3.2 Nutrient Uptake Inhibition

The inhibition of nutrient uptake in both UC157 and Gijnlim, with different rotational combinations is illustrated in **Fig. 6**. There were significant ($P \leq 0.05$) differences in the uptake of mineral nutrients N, P, K, Ca and Mg in the two asparagus varieties and their rotational combinations. The highest inhibition was found for P, followed by N, while the other nutrient widely varied in the different rotational combinations of asparagus varieties. The inhibition for uptake of total N, P, K, Ca and Mg ranged from 18 to 60; 29 to 83; 10 to 52; 8 to 48; 7 to 40 %, respectively. Generally, root residue might release allelochemicals into the soil which could be accumulated in the root and shoot systems and affect the nutrient uptake. In allelpathic treatments after the first replanting, the highest and lowest inhibitions for nutrient uptake were found in the UG and GU rotations, respectively. After the second replanting, the highest and lowest inhibitions were found in the UUG and GUG rotations, respectively. Similarly, in the autotoxic treatments, both the UU and UUU rotations showed significantly ($P \leq 0.05$) higher inhibition than the GG and GGG rotations. Therefore, varying the rotational combination could reduce the accumulation of the same autotoxins or allelochemicals or their same concentration. Rice (1984) also found that more allelochemicals are produced under the conditions of mineral nutrient deficiency without rotational cultivation. Fisher and Benson (1983) noted that while there were significant interactions ($P \leq 0.05$) between N and P for several aspects of asparagus growth at the higher concentrations of N, there was usually a positive response to increasing P. In this study, as the N uptake was reduced that might reduce the capability of detoxification by the increasing of allelochemicals which might be one reason for the uptake of P. This could be attributed to an increase in N immobilization following residue incorporation (Montoya-Gonzalez et al., 2009).

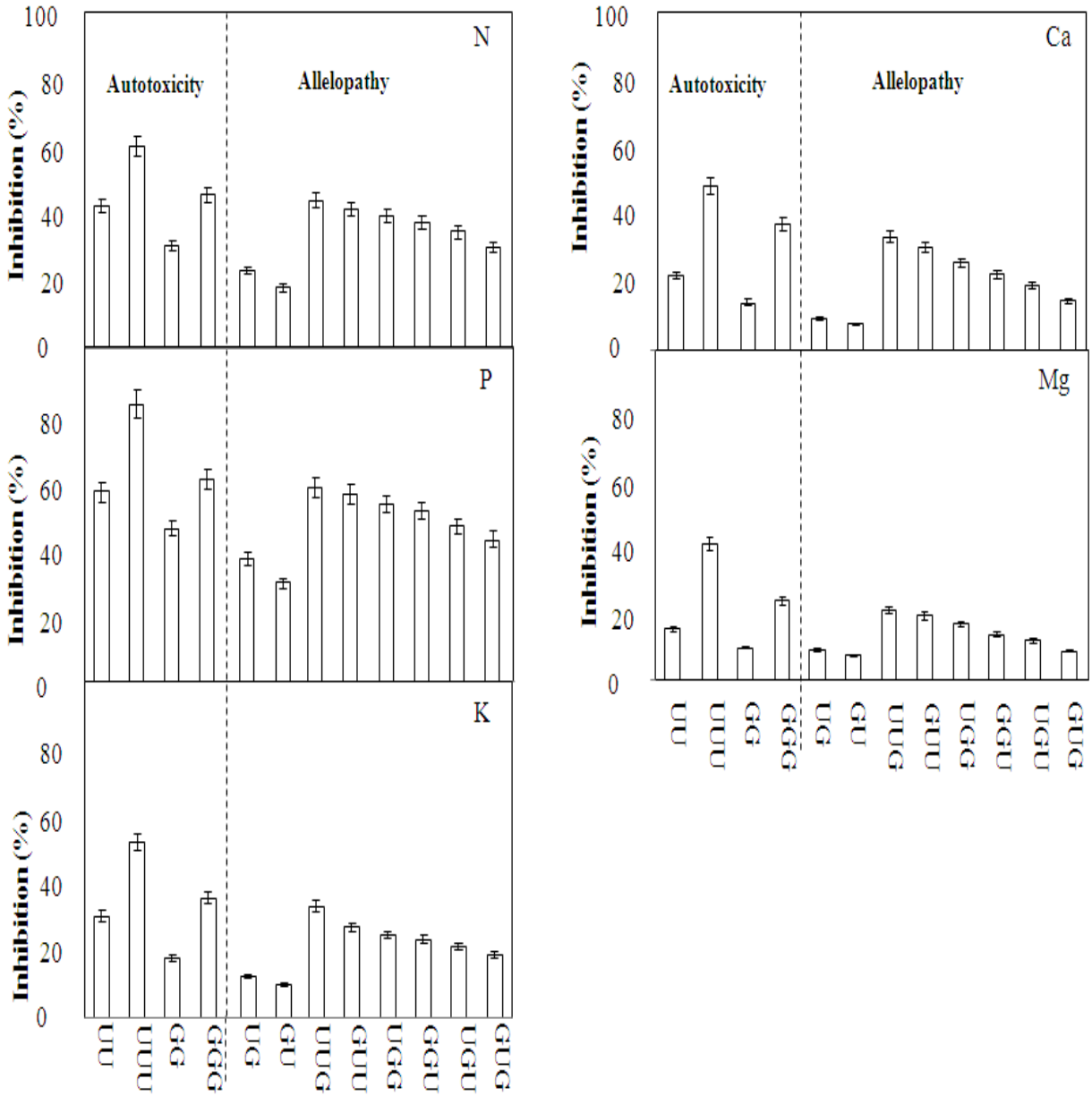


Fig. 6 The potential of allelopathy and autotoxicity between two asparagus varieties for the nutrient uptake (N, P, K, Ca and Mg) inhibition under different rotational combinations (RC)

Note: Inhibition (%) was calculated by using equation 1. U, UC157; G, Gijnlim; the order of the letters represents the varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Values in each combination are differ significantly (Tukey's protected multiple-comparison test, $P \leq 0.05$). Bars presented as mean ± SE, replication; $n = 3$.

Moreover, Wacker et al. (1990) reported about several allelochemicals were isolated and characterized from asparagus root tissue, including ferulic acids and which could inhibit P uptake in plant roots (Glass, 1973; McClure et al., 1978). Furthermore, plants have evolved special mechanisms to deal with nutrient deficiencies (Handreck, 1997), such as the release of allelochemicals and mycorrhizal symbiosis. Even though sterilized soil was used in this investigation; some microorganisms propagated along with the experimental period and replant treatments in the greenhouse which could be responsible for the nutrient uptake. Melloy et al. (2010) found that biomass of residue borne fungal pathogen was generally higher. Since, allelochemical activity could persist in soil with the same rotation and enhance microbial biomass that might be responsible for subsequent reduction of growth and nutrient uptake. However, the effect of previously grown plants on subsequent asparagus P content correlates well with the growth of asparagus. In contrast, the inhibition of K, Ca and Mg uptake was also followed the same tendency as those of N and P. But it might be necessary to determine the particular reason for that inhibition especially for P. Therefore, we are currently taking steps to exploring the specific causes of these problems and how to improve growth and nutrient uptake especially for P.

3.3 Effect of Subsequent Replanting on Physico-Chemical Properties of the Soil

Physico-chemical properties of the replanted soil with different rotational combinations are illustrated in **Table 3 (a)** and **(b)**. There were significant ($P \leq 0.05$) differences in the soil pH, EC, C: N ratio in the root residue amended soils compared to control soil (**Table 3 a**). However, pH in the amended and unamended (control) soils ranged from 3.5 to 6.8 and 6.6 to 7.0, respectively. EC of the root residue amended soils ranged from 0.7 to 2.3 $ds \cdot m^{-1}$, which is higher than that of unamended (0.4 to 0.6 $ds \cdot m^{-1}$) soils. Amount of C: N ratio in the amended and unamended soils ranged from 2.5 to 8.7 and 8.5 to 10.6, respectively.

Table 3 (a) Effects of root residue amendment on soil physic-chemical properties after the first, second (first replanting), and third (second replanting) plantings under different rotational combinations (RC)

PT	Treatment	RC	pH (H_20)	EC ($ds \cdot m^{-1}$)	C:N ratio
First P	Control	U	6.6±0.02b	0.6±0.01a	8.5±0.01b
	Control	G	7.0±0.01a	0.4±0.05b	10.6±0.03a
First RP	Aut.	UU	4.2±0.03c	1.2±0.03a	4.1±0.09c
		GG	4.6±0.01b	1.0±0.01b	6.4±0.02b
	Alle.	UG	5.5±0.02b	0.9±0.02b	6.9±0.01b
		GU	6.8±0.05a	0.7±0.03c	8.7±0.04a
Second RP	Aut.	UUU	3.5±0.06d	2.3±0.01a	2.5±0.09d
		GGG	3.7±0.01c	2.1±0.05b	2.9±0.08c
	Alle.	UUG	4.1±0.02b	2.0±0.03b	3.4±0.05c
		GUU	4.3±0.05b	1.8±0.02b	3.7±0.04b
		UGG	4.6±0.04b	1.7±0.05b	3.8±0.05b
		GGU	4.7±0.03b	1.6±0.00c	4.1±0.02b
		UGU	5.1±0.01b	1.4±0.04d	4.3±0.04b
		GUG	5.4±0.03a	1.3±0.01d	4.6±0.01a

Note: Data presented as mean ± SE, replication; *n*= 3. U, UC157; G, Gijnlim; the order of the letters represents the two varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Aut., Alle. and PT denotes toautotoxicity, allelopathy, and planting time, respectively. Lettering in the table refers to comparisons for the first and second replanting (Tukey's protected multiple-comparison test, $P \leq 0.05$).

There were significant ($P \leq 0.05$) difference between the two asparagus varieties and their rotational combinations after subsequent replanting in nutrient uptake; N, P, K, Ca and Mg (Table 3B). Amount of N, P, K, Ca and Mg ranged from 0.97 to 0.15, 0.33 to 0.04, 8.6 to 0.91, 0.93 to 0.12, 0.94 to 0.11 $mg \cdot g^{-1}$, respectively. Root residue amended treatment showed the decreasing trend in the soil nutrient uptake. Since pH was decreased with the increasing of planting time, ultimately soil became more acidic that might be caused by asparagus root residue (**Table 3a**). Thus, soil pH might have contributed to the higher or lower P and the other nutrient concentration. In addition, the alteration of C: N ratio in soils, leading to rapid assimilation of N by microorganisms, is known to reduce plant growth (Shilling et al., 1992) as well as nutrient uptake. This was probably because residue incorporation increased C substrate availability and stimulated dentrification (Baggs et al., 2003). In this study, EC was increased with the increasing of planting time. This parallels earlier reports that addition of residues or decomposing material of allelopathic plants into the soil can increase EC (Xuan et al., 2005). Moreover, allelochemical compounds in asparagus root residues might be activated in soil even in a fairly short time because of environmental conditions such as air, temperature, types and water content. Total N, P, K, Ca and Mg in soils reduced with the frequency are quite incomprehensible because of processes and properties of biogeochemical cycle of these elements in soils. Normally, soil total K, Ca and Mg chronically change, and mainly depend on weathering of parent material horizon. In this study, total N, P, Ca and Mg were lowered in soils with the application of plant residues **(Table 3b).** It suggested total N, P, Ca and Mg lost in very short period. Allelochemicals like benzoic, vanollic, cinamic and ferulic acids showed inhibition in P uptake; likewise, benzoic and trans-cinnamic acids reduced growth, lowered the amounts of soil P, K, Mg, Mn, Cl^{-1}, and SO_4^{-2} (Baziramakenga et al. 2005).

Table 3 (b) Effects of root residue amendment on soil nutrient after the first, second (first replanting), and third (second replanting) plantings under different rotational combinations (RC)

PT	Treatment	RC	N	P	K	Ca	Mg
					($mg \cdot g^{-1}$)		
First P	Control	U	0.85±0.04 b	0.28±0.0b	5.7±0.05b	0.81±0.02b	0.66±0.01b
	Control	G	0.97±0.01 a	0.33±0.02a	8.6±0.01a	0.93±0.00a	0.94±0.02a
First RP	Aut.	UU	0.51±0.05c	0.19±0.03c	1.8±0.01c	0.63±0.01c	0.34±0.02c
		GG	0.56±0.06b	0.21±0.01b	2.6±0.02b	0.70±0.02b	0.42±0.01b
	Alle.	UG	0.63±0.03b	0.23±0.00b	3.8±0.03b	0.77±0.03b	0.49±0.03b
		GU	0.68±0.02a	0.25±0.02a	6.3±0.04a	0.84±0.01a	0.72±0.01a
Second RP	Aut.	UUU	0.15±0.01d	0.04±0.01d	0.91±0.02d	0.12±0.03d	0.11±0.07d
		GGG	0.21±0.02c	0.06±0.01c	1.6±0.02c	0.15±0.03c	0.14±0.04c
	Alle.	UUG	0.25±0.08c	0.07±0.07c	1.7±0.04c	0.20±0.03c	0.17±0.03c
		GUU	0.29±0.06c	0.09±0.03c	2.4±0.08b	0.27±0.04c	0.20±0.02c
		UGG	0.34±0.04b	0.10±0.01b	2.7±0.04b	0.34±0.01b	0.24±0.05b
		GGU	0.39±0.03b	0.13±0.02b	2.8±0.04b	0.41±0.04b	0.27±0.01b
		UGU	0.44±0.09b	0.17±0.04b	2.9±0.03b	0.48±0.03b	0.31±0.03b
		GUG	0.49±0.07a	0.21±0.01a	3.1±0.01a	0.55±0.04a	0.37±0.02a

Note: Data presented as mean ± SE, replication; *n*= 3. U, UC157; G, Gijnlim; the order of the letters represents the two varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Aut., Alle. and PT denotes to autotoxicity, allelopathy, and planting time, respectively. Lettering in the table refers to comparisons for the first and second replanting (Tukey's protected multiple-comparison test, $P \leq 0.05$).

In addition, pH related to increases in nutrient availability and/or reductions in Al^{3+} availability (Lehmann et al., 2003; Rondon et al., 2007). Moreover, Major et al. (2010) reported that soil N (1.37 to 0.31 µg g·$soil^{-1}$), P (27.4 to 0.1 µg g·$soil^{-1}$), K (58.3 to 2.1 µg g·$soil^{-1}$), Ca (288.8 to 7.6 µg g·$soil^{-1}$) and Mg (91.8 to 5.5 µg g·$soil^{-1}$) showed decreasing trend due to 2 to 3 years continuous cropping. The present investigation also revealed that with the increasing of planting time soil nutrient showed decreasing trend. Therefore, it is likely that allelochemical compounds could be readily leached from the residues. After entering into the soil, allelochemicals are influenced by microorganisms (Inderjit, 2001). However, their negative effect depends greatly upon a variety of biotic and abiotic factors, soil type, presence of microorganisms and soil conditions, and further toxification and detoxification mechanisms in the soil (Blum et al., 1999). In addition, microbial degradation or transformation of allelochemicals in soil determines the expression of allelopathy and autotoxicity. Based on the observed results, the present study evidences that root residues of *Asparagus officinalis* suppress the growth of asparagus by releasing allelochemicals into the soil rhizosphere through alteration of soil nutrients.

3.4 Overall Physico-Chemical Characteristics Variability

Principle component analysis (PCA) of the total variation in different rotational combinations in the inhibitory effect of root residue amendment after the first, second replanting is shown in **Fig 7 (a)** and variability among physiochemical characteristics in **Fig 7 (b)**. **Fig 7 (a)** showed the combination, respectively. C: N ratio and pH showed very high factor loading, and dry weight of root is denoted by Dim 1. Dim 2 denotes the various physicochemical characteristics monitored throughout all replantings. The strong positive correlation among C: N ratio, N, and P suggest that C: N ratio might have been regulated for the P and N

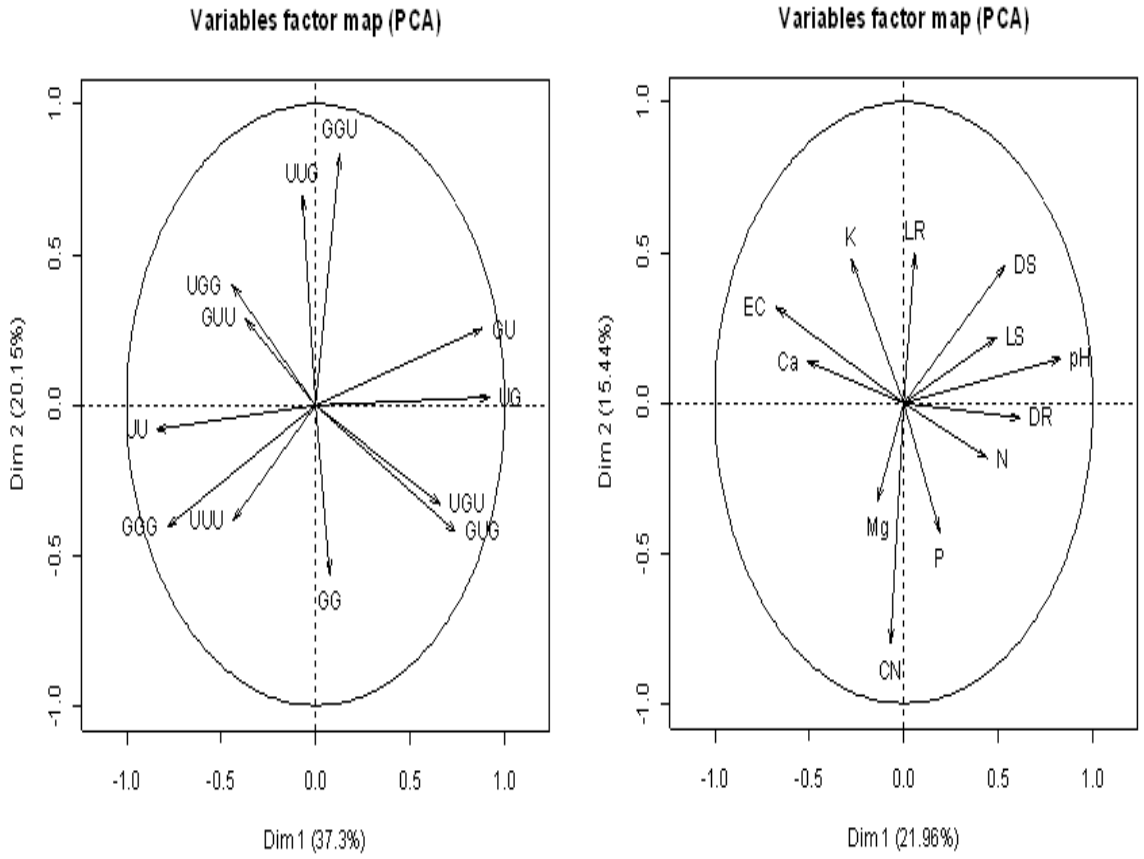


Fig. 7 (a) Variable factor map (PCA) for total variation in the inhibitory effects of root residue amendment after the first and second replanting in different rotational combinations (RC). **(b)** Variable factor map (PCA) for total variation in the physiochemical characteristics throughout all replanting

Note: LR, LS, DR and DS denote the length and dry weight of the root and shoot, respectively. U, UC157; G, Gijnlim; the order of the letters represents the varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively.

nutrients throughout the root residue amendment treatments, although negative correlation observed with EC. This result indicated that with an increased EC, the soils were nutrient poor with root residues amendment. This reconfirms the significant inhibitory effect of root residue amendment in all replanting stages as earlier described in this study. Moreover, among the physico-chemical interactions, N and P were positively correlated with the root and shoot growth, but negatively correlated with EC. There is now ample evidence that plant residue produces sufficient quantities of allelochemical substances to influence the growth and nutrient uptake of other plants (Batish et al., 2009). In the present study, results revealed that the rotational effect, therefore, is probably related to the mobilization of P as well as other nutrients through plant exudation; this inhibits the growth of any of the tested varieties. Based on the observed results, the present study evidences that root residues suppress the growth and nutrient uptake of asparagus and through alteration of soil nutrients and physicochemical properties. Thus, to improve asparagus growth and nutrient uptake, soil nutrient status and soil physicochemical properties, one is variety selection, the other is removal of plant residue to reduce allelopathic and autotoxic impacts in the ecosystem.

CHAPTER III

Allelochemicals inhibit the growth of subsequently replanted asparagus (*Asparagus officinalis* L.)

1. Introduction

Successive culture of the same crop on the same land for years causes soil sickness or replanting injuries (Tsuchiya 1990). Many studies have reported on yield reductions following its replanting in old asparagus fields (Hartung and Stephens 1983; Young and Chou 1985; Schofield 1991; Motoki et al. 2006). Possible reasons for these problems include allelopathy and autotoxicity. Allelopathy is the inhibitory and/or stimulatory effects of one plant, either microbial or higher plant, on another by the production of chemical substances that is released into the environment (Putnam and Tang 1986). Autotoxicity is a form of intraspecific allelopathy that occurs when a species releases chemical substances that inhibit or delay the germination and growth of plants of the same species (Putnam 1985). Allelochemicals produced by one crop species influence the growth, productivity, and yield of other crops or the same crop (Batish et al. 2007). For allelopathy to be an ecologically relevant mechanism influencing growth of plants, allelochemicals must accumulate and persist at phytotoxic levels and come in contact with the target plant (Inderjit 2005). Allelochemicals are considered to be one of the potentially important causes of the asparagus replanting problem (Putnam 1985). The phytotoxic compounds exuded by crop plants as root exudates could be employed to suppress growth in the vicinity (Kong et al. 2008).

Limited data have been reported on the identification of allelochemicals from root exudates. Fujii (1993) developed the plant box bioassay method, in which the source of the allelochemicals is planted in an agar growing medium and the test plants are planted in a grid surrounding this source, to evaluate allelopathic activity of allelochemicals exuded from roots.

Through the use of this technique, the allelopathic activities of more than 2000 kinds of plants have been evaluated. Growing plants in a nutrient solution allows exuded allelochemicals to accumulate and affect plant growth. Furthermore, wheat seedling allelopathy diminished after the addition of activated charcoal to the agar growth medium (Wu et al. 2000), indicating the involvement of allelochemicals exuded by wheat seedlings into the agar growth medium. Kitahara et al. (1972) isolated three organic compounds (growth regulators) from asparagus ferns: asparagusic acid (1, 2-dithiolane-4-carboxylic acid), dihydroasparagusic acid (β, β1-dimercaptoisobutyric acid); and (S)-acetyldihydroasparagusic acid (β-S-acetyl-β1-mercaptoisobutyric acid). Although these compounds strongly inhibit seedling growth, they were present at extremely low concentrations in asparagus tissues. In this chapter, a replanted culture system was used to identify the inhibitory component of the asparagus autotoxins which are responsible for growth inhibition and to quantify their toxicity on lettuce growth with bioassays.

2. Materials and methods

2.1 Asparagus replant

A replant culture system was employed to identify growth inhibitory activity of asparagus. Gijnlim crops have increased in area owing to its enhanced yield performance as compared to UC157 (Motoki et al. 2008). In this experiment, seeds of two asparagus cultivars, UC157 and Gijnlim of USA and European origin, respectively were obtained from a local commercial seed company (Sakata Seed Corporation, Japan). According to Fujii et al. (1992), the plant boxes (65 × 65 × 100 mm, Magenta, USA) were used as an evaluation of allelopathic activity in a bioassay method, filled with 250 ml autoclaved agar (Nacalai Tesque, Inc., Kyoto, Japan) medium in a clean bench (M-377, Sanyo, Osaka, Japan). After cooling the autoclaved agar 0.75% (w/v) (gelling temperature 30-31^{o}C) to 40^{o}C, it was poured into the plant box and,

kept on ice. The chapter was investigated the growth inhibition under laboratory condition **(Fig. 8)**. Therefore, to find the effects of growth inhibition in the same cultivar, after gelatinizing the agar, a total of 12 seeds of UC157 and Gijnlim were separately sown on agar medium in each plant box.

Prior to sowing, asparagus seeds were covered with a double layer of gauze and surface-sterilized in 70% ethanol and then rinsed in deionized water several times. Sterile culture techniques were adopted to rule out the possibility of interference by microorganisms in the culture medium. The plant boxes were wrapped with cling film between the cap and upper part of the box to prevent drying and also covered with aluminum foil to darken the roots. They were incubated at: 25° C; 12h light/12h dark; relative humidity 80% and 200 µmol m^{-2} s^{-1} intensity of light in growth chamber (MLR-351H; Sanyo, Tokyo, Japan) for 50 days. The control planting was the first planting of each cultivar of asparagus. After the growth of asparagus seedlings for 50 days, all the seedlings were uprooted from agar medium and the roots rinsed twice with 5 ml of distilled water to remove the residual agar. Investigating the growth inhibition with different cultivar by rotational cultivation, to find the effects of growth inhibition in the same cultivar, 12 seeds of both UC157 and Gijnlim were separately replanted (second planting, first replanting) in the same agar, the agar media that was used for first planting and left to grow in the same conditions as above for another 50 days. The third planting (second replanting) also followed the same procedures as above. All treatments were replicated three times.

2.2 Allelochemicals

The concentration of oxalic, succinic and tartaric acids in the medium were analysed by taking a sample of agar at a depth of 33 mm, which was the middle of depth at each plant box, according to the method of Fujii et al. (1991) with some modifications. The growth

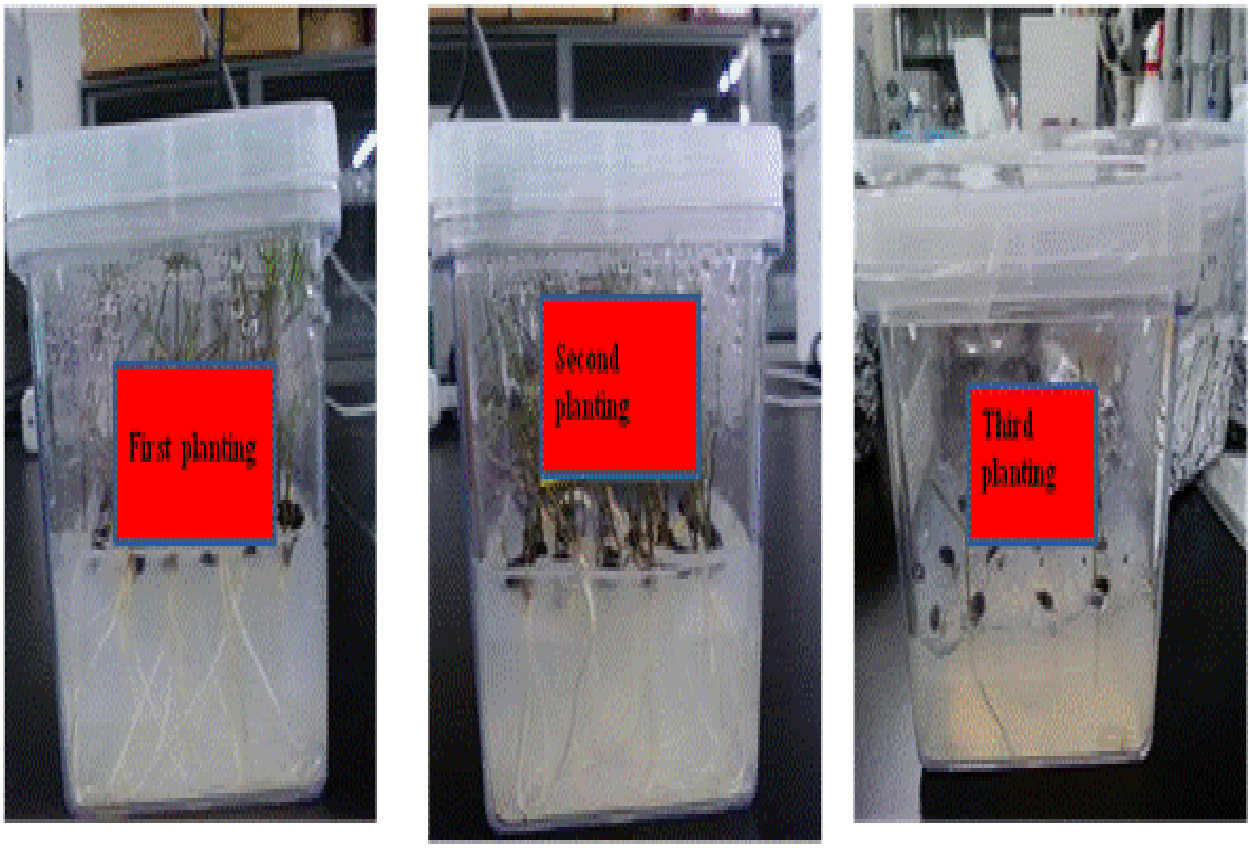


Fig. 8 Asparagus replanting under laboratory condition

medium was collected after each planting from each plant box for HPLC (ELITE LaCrom, Hitachi Co., Tokyo, Japan, column oven L-2350) analysis. The agar media was taken from the plant box in a liquid form using a porous tube (HPP- type, DIK-300B, Daiki Rika Kogyo Co., Ltd., porous part: resinous, size: porous part 2.5 x 1.5 x 50 mm, PVC tube 2.7 x 1.0 x 100 mm, pressure: 200 kPa, suction, in water, sucking in -1bar: 1 ml min^{-1}, syringe: 7 ml l^{-1} to 16hrs, about pF2.3, dead space: 0.5ml). This liquid samples along with the standard oxalic, succinic and tartaric acids in 20 µl quantities syringe were analysed with an HPLC and eluted with 0.1 M phosphate buffer. Concentrations in the sample were calculated by comparing peak areas of samples with those of the standards. Analysis condition was as follows: guard cartridge column: ODS - RS (6.0 LD X 15mm), pak DC- 613, temperature in column: 40°C. Quantitative analysis was performed by the internal standard method (Wu et al. 2001). All samples were run in triplicate.

2.3 Organic acid bioassay

Lettuce seedlings were selected to test the inhibitory concentration of identified organic acids according to Nishihara et al. (2005). To determine if an organic acid from asparagus roots was responsible for growth inhibition of lettuce, 0, 50, 100, 150 and 200 mg l^{-1} solutions of oxalic, succinic, and tartaric acids were prepared. Then, five seeds of lettuce (*Lactuca sativa* L.) were placed in 27 mm Petri dishes, containing a 27 mm filter paper (Adventec, Toyo Roshi, Ltd., Japan) moistened with 500 µl of the solution of each organic acid. In the control, seeds were placed in Petri dishes on filter paper, moistened with 500 µl of deionized water. The Petri dishes were incubated in a growth chamber under the conditions described above. After 5 days, root and shoot lengths (mm) were measured. This experiment treatment was replicated three times. Inhibition, expressed as a percentage, was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - Xt / Xc) \times 100 \qquad (1)$$

Where, Xc denotes the lengths of the roots or shoots of the control and Xt represents the mean values of the corresponding lengths of the treatments.

2.4 Plant growth measurements and statistical analysis

After removing the plants from the medium, plants were divided into roots and shoots and their fresh biomass (mg) was measured. Inhibition, expressed as a percentage, was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - Wt / Wc) \times 100 \qquad (2)$$

Where, Wc denotes the fresh biomass of the roots or shoots of the control and Wt represents the mean values of the corresponding fresh biomass in the second (first replanting) and third (second replanting) planting, respectively. Experimental data presented are the means of three replicates; Statistical analyses were executed using Stat View software. The percentage data was $\log_e$-transformed before analysis where necessary to equalize variances between treatments (Lam et al. 2012). Logarithmic approximation was used for trendline. The concentration of organic acids required to cause 50% inhibition (IC_{50} value) of lettuce growth was calculated using an exponential function (Nishihara et al. 2005).

3. Results & discussion

3.1 Growth

The percentage of asparagus growth inhibition after the first and second replanting is shown in **Fig. 9**. Root and shoot growth inhibition were significantly higher in UC157 than in Gijnlim, with the second (**Fig. 9b**) replanting showing a higher inhibition than the first (**Fig. 9a**). Root (81%) growth was more severely inhibited than shoot (19%) growth, since the root was more closely in contacted with the growing medium. Figure 1 illustrates the growth of

the asparagus seedlings was retarded under replanting conditions. This implied that asparagus plants contain growth inhibitors that are capable of reducing the growth of its own seedlings under replanting condition. Increased autotoxicity in asparagus root exudates due to accumulation of allelochemicals from subsequent replantings might have been responsible for asparagus growth inhibition (Young and Chou 1985). Allelochemical compounds including amino acids, organic acids, sugars, phenolic acids, and other secondary metabolites, serve as an important medium of root-based interactions with other microorganisms including bacteria, actinimycetes, pathogens, fungi, and insects in the growing media (Walker et al. 2003). Therefore, with increased number replanting, the allelochemicals could become more concentrated and cause severe growth inhibition. Similar findings were reported for *Chenopodium murale* grown under laboratory condition where the root and shoot growth was reduced by 44 and 32%, respectively (Batish et al. 2007). Inhibitory effects of allelochemicals varied with cultivar (Chung et al. 1997). This result revealed that the growth inhibition by allelochemicals would be expected to reduce the competitiveness of the affected plants under replanting condition.

3.2 Allelochemicals

To understand chemically mediated allelopathy and autotoxicity in managed crop ecosystems, identification of allelochemicals or phytotoxins and their potential action mechanisms with replant problem is required. Therefore, in this study, allelochemicals, which could be exuded by asparagus roots, and their concentrations in the agar growing media were analysed. Asparagus cultivars exuded differential concentrations of oxalic, succinic and tartaric acids into the growth medium after replantings (**Fig. 10**). After the first planting (**Fig. 10a**), UC157 and Gijnlim both produced small amount of organic acids.

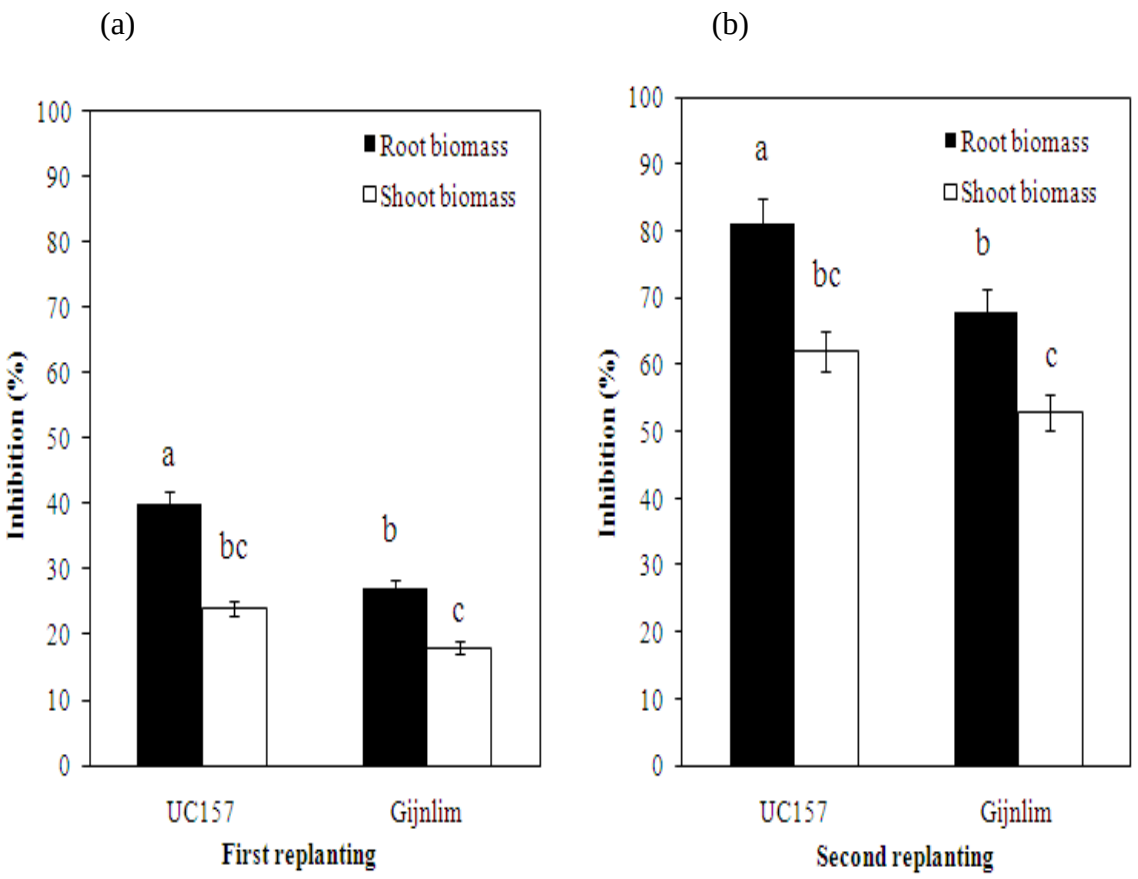


Fig. 9 Percentage of inhibition of asparagus seedling growth at first (a) and second (b) replantings. Tukey's protected multiple-comparison test at $p< 0.05$ was used to compare means Bars presented as mean ± SE.

The average levels of organic acids exuded by asparagus seedlings into the agar growth medium increased with the replanting times. Both UC157 and Gijnlim produced higher concentrations of oxalic, succinic and tartaric acids in the second (**Fig. 10c**) than in the first replanting (**Fig. 10 b**) although both cultivars had the tendency to produce similar ratios of the three acids. Organic acids released in asparagus root exudates into the medium could be responsible for the replanting problem (**Fig. 10**). Many organic compounds are inhibitory to growing plants (Rice 1984). Wu et al. (2001) noted that sorghum plants exuded sorgoleone, a hydroquinone that is quickly oxidized to a benzoquinone, which can inhibit weed growth at extremely low concentrations (0.01-0.125 mM). For allelopathy to be an ecologically relevant mechanism influencing the growth of plants, allelochemicals must accumulate and persist at phytotoxic levels and come in contact with the target plant (Inderjit 2005). Moreover, Miller et al. (1991) reported on allelopathically active fractions, tested by a curly cress germination bioassay, included ferulic, isoferulic, malic, citric, fumaric, caffeic (CA) and methylenedioxycinnamic (MDA) acids, and noted that no single compound was responsible for the allelopathic activity of asparagus extracts, although MDA did severely inhibit curly cress growth at concentrations of 25 ppm or above. In addition, organic compounds are known to be of great significance in allelopathy (Inderjit 1996). The present study demonstrated that asparagus seedling roots are able to exude varied amounts of organic acids into the agar growth medium and could be responsible for growth inhibition of asparagus cultivars.

3.3 Bioassay

Concentrations of oxalic, succinic and tartaric acids inhibitory to lettuce seedlings (IC_{50}) were determined (**Fig. 11**). The data support that the asparagus growing media contained allelochemicals that exert an allelopathic effect on lettuce.

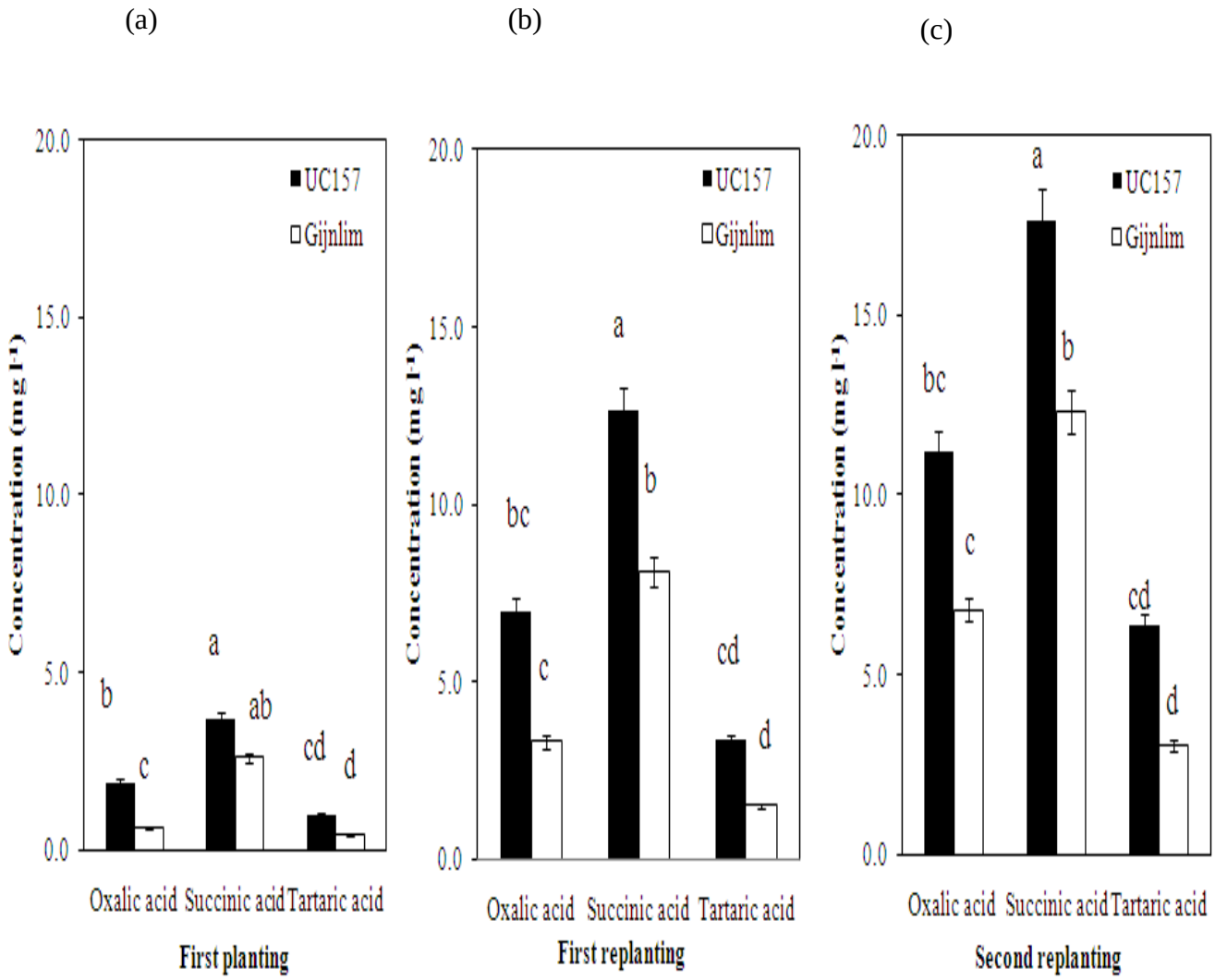
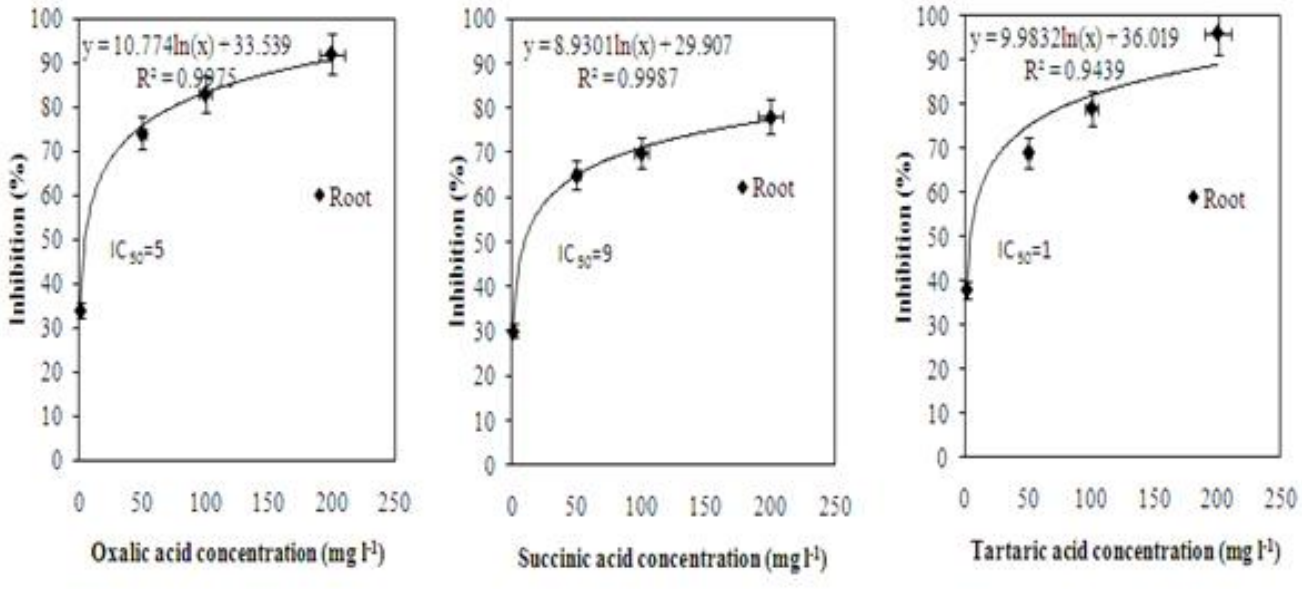


Fig. 10 Concentration (mg l^{-1}) of organic acids at first planting (a) and first (b) and second (c) replantings. Tukey's protected multiple-comparison test at $p < 0.05$ was used to compare means Bars presented as mean ± SE.

The inhibitory IC_{50} for lettuce shoot growth (**Fig. 11 b**) was higher than for root (**Fig. 11a**). Figure 14 illustrates the asparagus growing media inhibitory to lettuce seedlings (IC_{50}). Kalinova et al. (2007) determined that 4- hydroxyacetophenone inhibited the growth of eight tested plants, and IC_{50} ranged from 378 to 1915µM, depending on the plant parts and species tested. In the present study, results show that allelochemicals were more effective in inhibiting lettuce root growth (**Fig. 11 a**), but less effective on shoot growth (**Fig. 11 b**). Release of phytotoxic compounds could also be affected by type of plant parts (Batish et al. 2007). Differences in phytotoxicity of various plant parts have been reported previously (Rice 1984; Putnam and Tang 1986). Both of these factors could potentially regulate the release of allelopathic compounds (Shilling et al., 1992). Organic compounds are major allelochemicals causing growth inhibition and might be responsible for replanting problems even at low concentrations. Gerig and Blum (1991) reported that mixtures of phenolic acids showed a more than additive inhibitory effect on cucumber growth compared with

individual acids. The varying degree of inhibition with differential responses to the allelopathic and autotoxic compatibility would be valuable in predicting the potential growth inhibition of subsequent asparagus cultivation. Therefore, steps are currently being taken to explore the specific causes of these problems and how to improve growth with replanting.

(a)



(b)

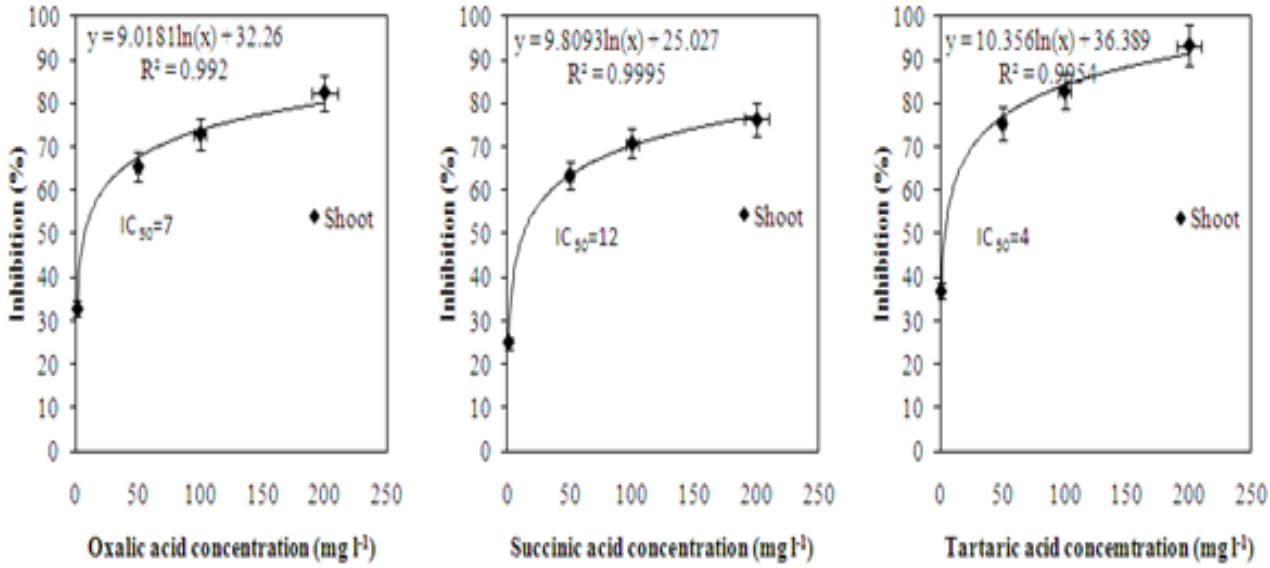


Fig. 11 Inhibitory concentrations (IC_{50}) for lettuce root (a) and shoot (b) growth. Points presented as mean ± SE.

CHAPTER IV

Inference of allelopathy and autotoxicity to varietal resistance of asparagus (*Asparagus officinalis* L.)

1. Introduction

Asparagus has shown a marked decline in productivity after many years of continuous replanting. There are many reasons for the replanting problems such as disease, insect, pathogen, microorganism, soil physical and chemical properties, reduced number and diameter of stems, crowns and roots, and collapse of storage roots (Blok and Bollen, 1993). One of the potential reasons for the problems was included allelopathy and autotoxicity. In previous chapter we already described about allelopathy and autotoxicity. Additionally, the chemical interactions between varieties within the same crop species can be classified as 'varietal allelopathy' and 'varietal autotoxicity' (Wu et al., 2007). Allelochemicals are considered to be one of the potentially important causes of the asparagus replanting problem (Putnam, 1985). Allelochemicals produced by one crop species influence the growth, productivity, and yield of other crops or the same crop (Batish et al., 2007) and the growth and activity of soil fungi and bacteria (Blok and Bollen, 1993). Allelopathy and autotoxicity are closely linked to environmental stresses such as nutrient deficiency (Young, 1984). There is no information on the varietal allelopathic and autotoxic effects of asparagus in a continuous replanting system with different rotational patterns. Therefore, the aim of this study was firstly, to evaluate varietal resistance of asparagus to allelopathy and autotoxicity with different rotational combinations under laboratory conditions. Secondly, to identify the potential allelochemicals released from asparagus root exudates in two different varieties.

2. Materials and methods

2.1 Planting materials

The seeds of two asparagus varieties; UC157 and Gijnlim of USA and European origin, respectively, were procured from a local commercial seed company (Sakata Seed Corporation, Yokohama, Japan).

2.2 Assessment of varietal allelopathy and autotoxicity

A replant culture system was employed to identify growth inhibitory activity of asparagus. The plant boxes (65 × 65 × 100 mm, Magenta, New Milford, CT, USA) were filled with 250 ml autoclaved agar (Nacalai Tesque, Inc., Kyoto, Japan) medium in a clean bench (M-377, Sanyo, Osaka, Japan). After cooling the autoclaved agar 0.75% (w/v) (gelling temperature 30-31^{o} C) to 40 ^{o} C, it was poured into the plant box and, kept on ice. After gelatinizing the agar, a total of 12 seeds of UC157 and Gijnlim were separately sown in each plant box. Prior to sowing, asparagus seeds were covered with a double layer of gauze and surface-sterilized in 70% ethanol and then rinsed in deionized water for several times. Sterile culture techniques were adopted to rule out the possibility of interference by microorganisms in the culture medium. The plant boxes were wrapped with cling film between the cap and upper part of the box to prevent drying and also covered with aluminum foil to darken the roots. They were incubated at: 25 °C; 12-h light/12-h dark; relative humidity 80% and 200 µmol m^{-2} s^{-1} intensity of light in growth chamber (MLR-351H; Sanyo, Tokyo, Japan) for 56 days. First planting of each variety served as the control. After 56 days, seedlings were harvested and seeds of both UC157 and Gijnlim were then replanted in the same agar (the agar media that was used for first planting) for the first replanting (second planting) and left to grow under growth chamber (conditions are same as above) for another 56 days. The above procedures were done under the clean bench to reduce contaminations. The third (second replanting) planting also followed the same procedures as above. At the end of each planting, 12 seedlings average values (fresh or dry mass of the roots or shoots) were calculated, termed

as a one seedling value (fresh or dry mass of the roots or shoots) for each treatment. All treatments were replicated three times.

Inhibition, expressed as a percentage, was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - Xt/Xc) \times 100 \qquad (1)$$

Where Xc denotes the fresh or dry mass of the roots or shoots of the control and Xt represents the mean values of the corresponding fresh or dry mass in the second (first replanting) and third (second replanting) planting. The above equation was used to compare the performance of the two varieties UC157 (U) and Gijnlim (G) in the following rotational combination patterns for varietal allelopathy: UG, GU (first replanting) and UGU, UUG, UGG, GUG, GGU, GUU (second replanting) and autotoxicity: UU, GG (first replanting) UUU, GGG (second replanting), respectively.

2.3 Measurements of plant growth and nutrient uptake

After harvesting at the end of each planting, all seedlings were carefully separated into roots and shoots, thoroughly cleaned, blotted dry between absorbing paper and their fresh mass were measured. Root and shoot dry masses were measured after oven drying at 70°C for 72h. To determine nutrient uptake, all dried root and shoot parts were combined and ground to a fine powder using a stainless ball mill. Total nutrients N, P, K, Ca and Mg were analyzed using standard procedures. Total N content was determined by the dry combustion method with an automated C-N coder (Model MT 700; Yonaco, Tokyo, Japan), total major mineral nutrients K, Ca and Mg were determined after digestion with a H_2O_2-H_2SO_4 mixture. Total P in the digested mixture was determined calorimetrically with a spectrophotometer (Model U-2001, Hitachi Co., Tokyo, Japan) using the phosphomolybdate blue method (Murphy and Riley, 1962). The total amounts of K, Ca, and Mg were determined with an atomic absorption

spectrophotometer (Model Z-8100, polarized Zeeman; Hitachi Co., Tokyo, Japan). Inhibition, expressed as a percentage, for nutrient uptake was calculated by using equation 1.

2.4 Identification of potential allelochemicals

The separation and identification procedures of selected allelochemicals were conducted according to a method of Fujii et al. (1991) with some modifications. Three standard chemicals including oxalic acid, succinic acid, tartaric acid at concentrations of 0, 50, 100, 150, 200 and 250 mgL^{-1} and asparagus root exudate samples were injected in 20 µl quantities and subjected to HPLC (ELITE LaCrom, Hitachi Co., Tokyo, Japan, column oven L-2350) analysis. Concentrations in the sample were calculated by comparing peak areas of samples with those of the standards. Conditions of HPLC were as follows: ODS - RS pack DC- 613, guard cartridge column (6.0 mm LD X 15mm), temperature in column: 40°C, equipped with ECD (Electro Conductivity Detector) and eluted with 0.1 M phosphate buffer. This experiment was conducted with three replications.

2.5 Statistical analysis

Experimental data presented are the means of three replicates; Statistical analyses were executed using Stat View software. The percentage data was $\log_e$-transformed before analysis where necessary to equalize variances between treatments (Lam et al. 2012). Tukey's protected multiple- comparison test (at $P < 0.05$) was used to compare the percentage of inhibition in root and shoot growth. Principle component analysis (PCA) was performed using XLSTAT 2011 to clarify total data variability with respect to co-relationships between growth, nutrient uptake, and allelochemicals characteristics after the first and second replantings. Moreover, each rotational combination was treated as categorical data, converted

to dummy variables and PCA performed along with other variables. Due to very low cumulative contribution ratio, some dummy variables were omitted.

3. Results and Discussion

3.1 Varietal resistance to allelopathy and autotoxicity

The potential of allelopathy on root and shoot growth pattern between two asparagus varieties with different rotational combinations under laboratory conditions are illustrated in **Table 4**. There were significant ($p< 0.05$) differences in root and shoot growth patterns between the two varieties when grown under the different rotational combinations. The second replanting showed a higher inhibition in growth than the first replanting. Root growth was more severely inhibited than shoot growth in both varieties since the roots were directly in contact with the growing media. After the first replanting, the highest and lowest inhibitions for root and shoot growth were found in the UG and GU rotations, respectively. After the second replanting, the highest and lowest inhibitions were found in the UUG and GUG rotations, respectively. This combined result indicated that UC157 might be producing more allelochemicals than Gijnlim and that might be responsible for the inhibited growth. Allelochemical compounds including amino acids, organic acids, sugars, phenolic acids, and other secondary metabolites, serve as an important medium of root-based interactions with other microorganisms including bacteria, actinimycetes, pathogens, fungi, and insects in the growing media (**Fig. 12 a** and **b**). Therefore, with increased

Table 4 Percent (%) inhibition in asparagus seedling growth due to autotoxicity or allelopathy for UC157 (U) or Gijnlim (G) after the first and second replantings with different rotational combinations

			Fresh Mass		Dry Mass	
		RC	Root	Shoot	Root	Shoot
First replanting	Autotoxicity	UU	41±1.3 a	36±0.9 a	40±1.9 a	35±3.2 a
		GG	21±0.8 b	17±0.5 b	26±5.6 b	19±2.4 b
First replanting	Allelopathy	UG	16±3.1 c	12±1.9 c	24±1.3 b	14±1.3 b
		GU	8±2.4 c	5±0.4d c	15±7.7 c	13±1.5 c
Second replanting	Autotoxicity	UUU	77±0.2 a	61±1.4 a	73±2.0 a	70±1.5 a
		GGG	46±0.1 b	41±0.3 b	47±0.4 b	44±0.8 b
Second replanting	Allelopathy	UUG	43±0.3 b	37±0.6 b	42±1.1 b	41±0.7 b
		GUU	41±0.2 b	35±0.4 b	40±0.8 b	38±0.7 b
		UGG	37±0.4 b	33±0.7 b	37±1.8 b	34±1.1 b
		GGU	35±0.1 b	30±0.6 b	33±0.6 b	30±2.6 b
		UGU	31±0.4 b	26±0.8 b	30±0.3 b	27±2.6 b
		GUG	26±0.8 b	21±0.4 b	29±0.5 b	22±0.4 b

Note: Listed fresh mass and dry mass suggests the values are actual fresh mass (g) and dry mass (g). Inhibition (%) was calculated by using equation 1. U = UC157; G = Gijnlim; the order of the letters represents the two varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Lettering (a, b, c) in the table refers to comparisons for the first and second replantings separately (Tukey's protected multiple-comparison test, $p < 0.05$) and data presented as mean ± SE, replication ($n = 3$).

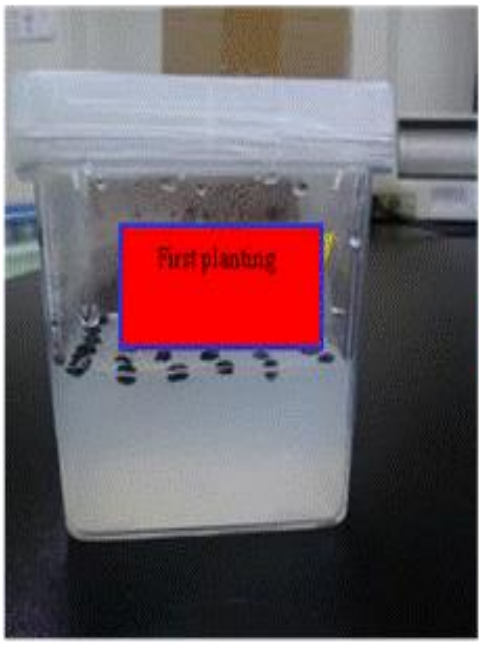


Fig. 12 (a) First replanting media

Fig. 12 (b) Second replanting media

number replanting, the allelochemicals could become more concentrated and cause severe plant growth inhibition. Growth inhibition by allelochemicals would be expected to reduce the competitiveness of the affected plants and the selection of specific variety under different rotational combinations may increase the security of future plant performance. **Table 4** illustrates the potential of autotoxicity on root and shoot growth pattern after the first and second replantings in the same rotation with each variety. In autotoxic combinations, both the UU and UUU rotations showed significant ($p< 0.05$) higher growth inhibition when compared to the GG and GGG rotations. Increased autotoxication by asparagus root exudates due to accumulation of allelochemicals from subsequent replantings might have been responsible for growth inhibition (Young and Chou, 1985). The present results indicated that asparagus is an auto-inhibited species which significantly inhibits the growth of seedlings its own species. Aqueous extract of living asparagus roots were strongly inhibitory to the growth of asparagus (Hazebroek et al., 1989).

3.2 Nutrient uptake inhibition

The percentage inhibition for the asparagus nutrient uptake in both UC157 and Gijnlim, with different rotational combinations is presented in **Fig. 13**. There were significant ($p< 0.05$) differences in the uptake of mineral nutrients such as N, P, K, Ca and Mg between the two varieties grown under the different rotational combinations. The percentage inhibition for total N, P, K, Ca and Mg concentration in the plant ranged from 11- 55; 14- 71; 8-45; 6-41; 5-33 %, respectively. The highest percentage inhibition was found for P, followed by N, while the others nutrients widely varied in the different rotational combinations of asparagus varieties. After the first replanting, the highest and lowest inhibitions for nutrient uptake under allelopathic

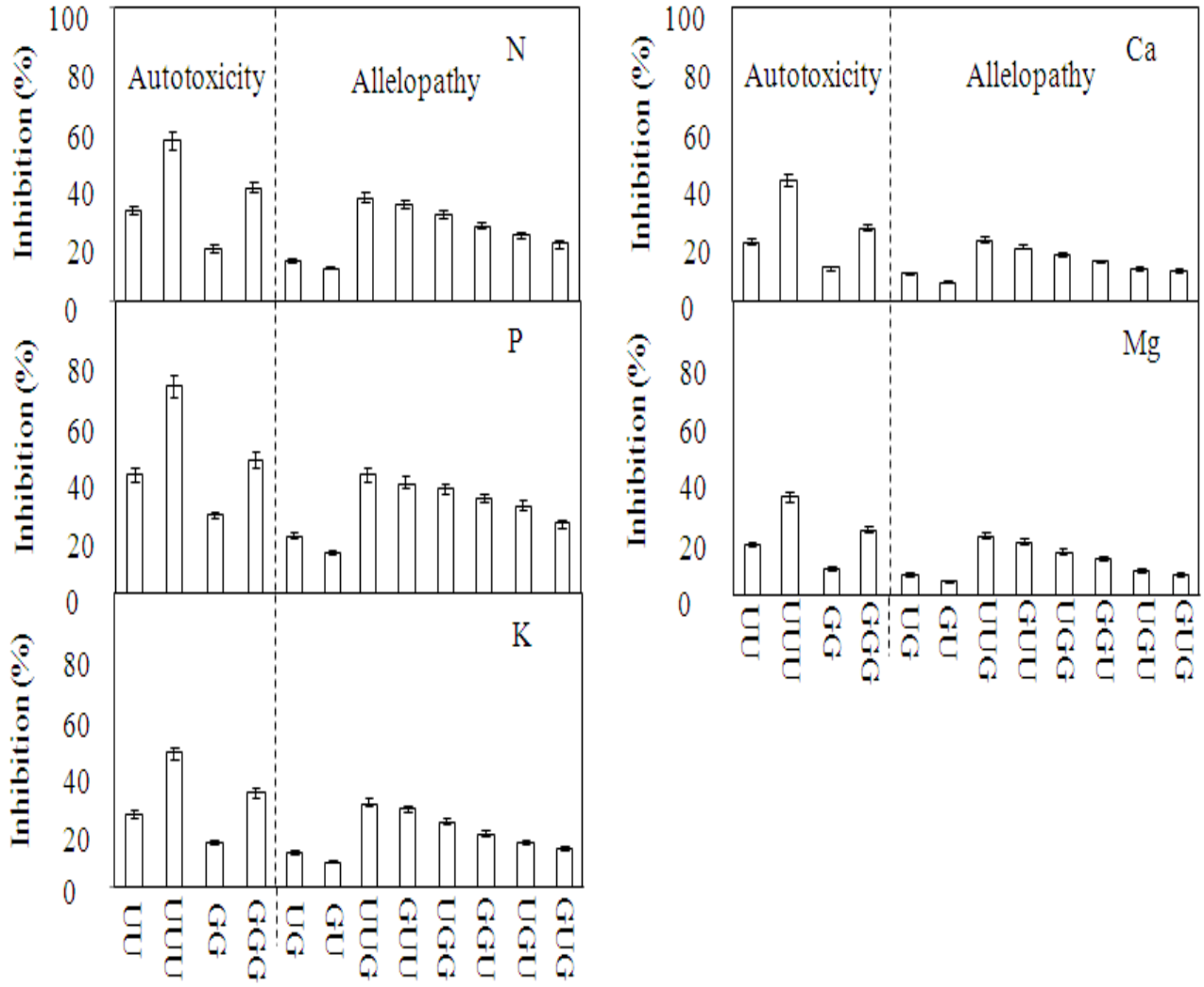


Fig. 13 Percent (%) inhibition in asparagus seedling nutrient uptake due to autotoxicity or allelopathy after the first and second replantings with different rotational combinations.

Note: Listed nutrient uptake suggests the values are actual concentrations (mg g^{-1}) of N, P, K, Ca and Mg in asparagus seedlings. Inhibition (%) was calculated by using equation 1. The order of the letters (U and G) represents the varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Values in each combination are differ significantly (Turkey's protected multiple-comparison test, $p< 0.05$), and data presented as mean ± SE, replication ($n = 3$).

combinations were found in the UG and GU rotations, respectively. After second replanting, UUG and GUG rotations showed the highest and lowest inhibitions, respectively. Similarly, in the autotoxic combinations, the UU and UUU rotations showed significant ($p< 0.05$) greater percentage inhibition than the GG and GGG rotations. Involvement of autotoxins from root exudates of previous asparagus crops was evaluated for the replanting problem (Young and Chou, 1985). Therefore, varying the varietal rotational combination could reduce the accumulation of the same autotoxins or allelochemicals or their concentrations. Allelochemicals like benzoic, vanollic, cinamic and ferulic acids have been shown inhibition to P uptake; likewise, benzoic and trans-cinnamic acids reduced root and shoot dry biomass and lowered the amounts of P, K, Mg, Mn (Baziramakenga et al., 2005). In this study, the effect of previously grown plants on subsequent asparagus P content correlates well with the growth of asparagus. In contrast, the percentage inhibition of K, Ca and Mg concentration in tissue followed the same tendency as those of N and P. But it might be necessary to determine the particular reason for that inhibition especially for P under different rotational combinations with asparagus varieties.

3.3 Identification of potential allelochemicals

Table 5 illustrates the concentration of organic acids from root exudates in both UC157 and Gijnlim, after two replantings with different rotational combinations. There were significant ($p<0.05$) differences among the production of allelochemicals between the two varieties under different rotational combinations. On average, UC157 produced more oxalic, succinic and tartaric acids than Gijnlim; although both varieties had the tendency to produce same allelochemicals. After the first replanting, the highest concentrations of oxalic, succinic and tartaric acids were found in the UG rotation and the lowest concentrations were in GU

Table 5 Effects of the identified organic acids at different concentrations (mg L^{-1}) on growth and nutrient uptake of asparagus after the first and second replantings.

		RC	Oxalic acid	Succinic acid	Tartaric acid
			(mgL^{-1})		
First planting	Control	U	1.1±0.6 c	1.8±0.9 c	1.2±0.3 c
First planting	Control	G	0.2±0.1 c	1.3±0.5 c	0.7±0.1 c
First replanting	Autotoxicity	UU	5.3±0.8 a	11.4±1.4 a	7.5±1.4 a
		GG	4.0±0.0 a	8.5±0.1 a	5.4±1.0 a
First replanting	Allelopathy	UG	2.9±0.1 b	7.4±0.4 a	4.6±0.6 a
		GU	0.6±0.2 c	2.2±0.3 b	1.3±0.3 b
Second replanting	Autotoxicity	UUU	7.6±1.7 a	17.1±0.1 a	11.1±2.2 a
		GGG	5.7±0.2 a	13.8±0.8 a	9.3±0.1 a
Second replanting	Allelopathy	UUG	4.5±0.1 a	10.2±0.4 a	8.2±0.8 b
		GUU	3.5±0.3 a	8.0±0.2 a	6.7±0.6 b
		UGG	2.9±0.3 b	6.6±0.8 a	4.6±0.3 b
		GGU	2.2±0.2 b	5.3±0.5 b	4.4±0.5 b
		UGU	1.8±0.4 b	4.2±0.4 b	2.9±0.9 c
		GUG	1.3±0.1 c	2.5±0.1 c	1.8±0.5 c

Note: U = UC157; G = Gijnlim; the order of the letters represents the two varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively. Lettering (a, b, c) in the table refers to comparisons for the first and second replantings separately (Tukey's protected multiple-comparison test, $P < 0.05$) and data presented as mean ± SE, replication ($n = 3$).

rotation. After the second replanting, the highest and lowest concentrations of those allelochemicals were found in the rotational combination UUG and GUG, respectively. These results reveal that allelochemicals produced by asparagus root released into the growing medium could be responsible for the replanting problem. For allelopathy to be an ecologically relevant mechanism influencing the growth of plants, allelochemicals must accumulate and persist at phytotoxic levels and come in contact with the target plant (Inderjit, 2005). Quantitative and qualitative differences in allelochemicals would be likely to lead to differential allelopathic and autotoxic effects which might be responsible for variations in growth and nutrient uptake.

In autotoxic combinations, there were significant ($p < 0.05$) differences in the concentrations of oxalic, succinic and tartaric acids between the two varieties and their rotational combinations (**Table 5**). Both the UU and UUU rotations produced higher concentrations of these acids as compared to the GG and GGG rotations, respectively. The greatest growth and nutrient uptake inhibitions were also found in the UU and UUU rotational combinations. This suggests the inhibited growth and nutrient uptake could be caused by differences in the allelochemical substances released from asparagus root exudates. Autotoxicity of root exudates is one of the important features for understanding replanting problems in agroecosystem as it represents one of the largest direct inputs of allelochemicals into the rhizosphere environment with potent biological activity and great variation in chemical components (Inderjit, 1996). Thus, in this study, results showed that organic compounds are major allelochemicals causing varietal allelopathy or autotoxicity, which might be responsible for growth and nutrient uptake inhibitions even at a low concentration.

3.4 Overall physico-chemical characteristics variability

Principle component analysis (PCA) was used to differentiate the variation in different rotational combinations on the inhibitory effect of allelochemicals on the growth and nutrient uptake after the first, second replanting (**Fig. 14a**.) and variability among physiochemical characteristics in **Fig. 14 (b)**. **Fig. 14 (a)** showed the correlation ship within the same replanting treatments among different rotational combination, whereas, GU and UG rotational combinations showed the strong positive correlation, although negatively correlated with UU, GGG and UUU rotational combinations. The strong positive correlation among dry mass of root, N, and P suggested that N might have been regulated the dry mass and P nutrient, although negative correlation observed with oxalic, succinic and tartaric acids. This reconfirms the significant inhibitory effect of allelochemicals in all replanting stages as earlier described. In this study, results reveal that the rotational effect, asparagus seedling roots are able to exude varied amounts of organic acids into the growth medium and could be responsible for variations in asparagus varieties growth and nutrient uptake.

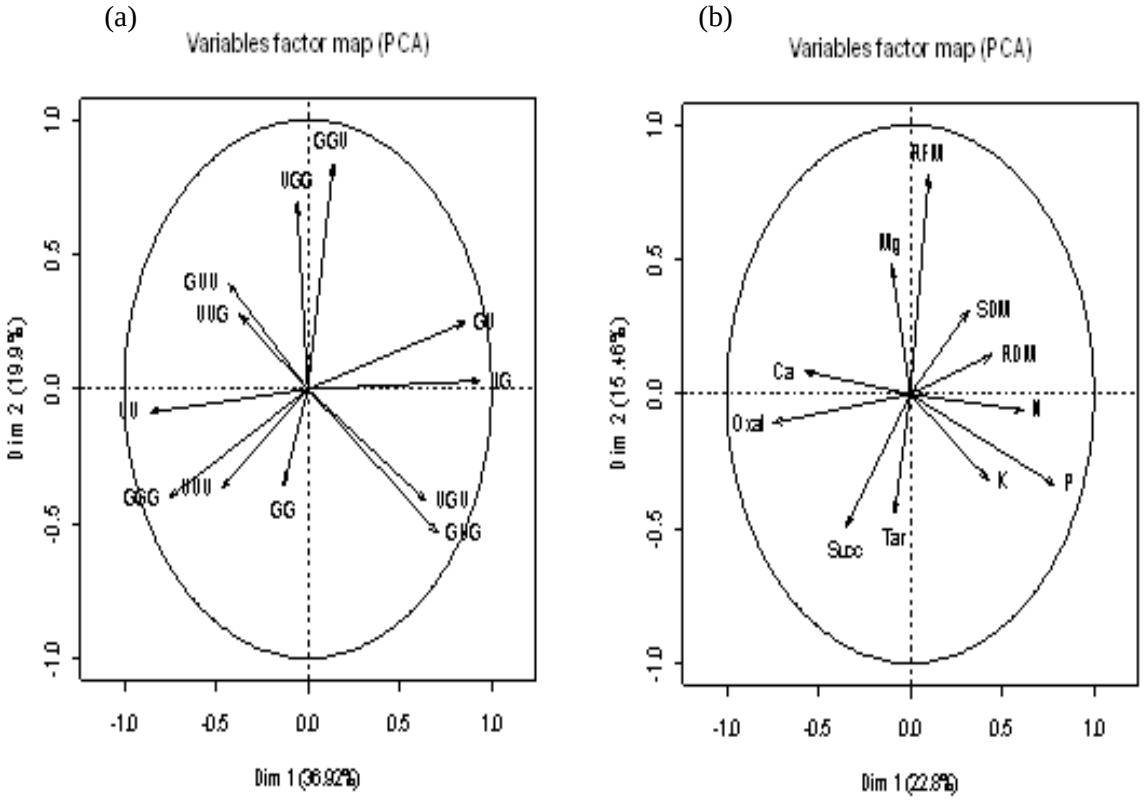


Fig. 14 (a) Variable factor map (PCA) for total variation in different rotational combinations after the first and second replantings. Dim 1 and Dim 2 explain 36.92 and 19.9 % of the variation observed, respectively. U = UC157; G = Gijnlim; the order of the letters represents the varieties used in the first, second (first replanting), and third (second replanting) plantings, respectively.

(b) Variable factor map (PCA) for total variation in the physiochemical characteristics throughout all replantings. Dim 1 and Dim 2 explain 22.8 and 15.45 % of the variation observed, respectively. Here, RFM = root fresh mass, RDM = root dry mass, SDM = shoot dry mass, Oxal = oxalic acid, Succ = succinic acid and Tar = Tartatic acid.

CHAPTER V

Activated carbon and phosphorus reduces the negative impact of root residue amendment on subsequent asparagus (*Asparagus officinalis* L.) growth

1. Introduction

Root residues entering into the soil can affect growth and yield through changes in microbial activities and nutrient immobilization or availability (Hungate et al., 1997). Batish et al. (2009) reported that phenolics were present in root residue amended soils, and growth reduction in root residue amended soils was concomitant with the amount of phenolic compounds. Crop residues added to soils have been shown to reduce the recovery of fertilizer (Kongchum et al., 2007). In addition to being scarce, P is also poorly available to plants that are not adapted to these conditions. In general, under low P availability plant biomass accumulation decreases and root morphology is modified (Lambers et al., 2006). Phosphorus (P) is a critical macronutrient required for numerous functions in plants and is one of the limiting factors for plant growth due to its rapid immobilization by soil organic and inorganic components (Richardson et al., 2009). The evolutionary adaptations of plants to P deficiency include responses that help them enhance the soil P availability, increase its uptake and improve the use efficiency of P within a plant (Lambers et al., 2006).

Activated carbon (AC), with its large surface area and pore volume, as well as its polarity, has tremendous adsorptive capacity (Lau et al., 2007). AC adsorbs the putative allelochemical due to its great affinity for organic molecules and has very little affiliation for inorganic molecules (Batish et al., 2007). AC has been used to substantially reduce negative effects of plant–plant competition by adsorption of allelopathic substances excreted by plant

roots (Kumar et al., 2009). Furthermore, AC additions would also be expected to increase the growth of plants (Kulmatiski and Beard, 2006). AC has ability to strongly adsorp organic compounds, is often used as a soil amendment for detoxification purposes. One of the establishment of seedling technique was reported the utilization of AC for absorbing the allelopathic compounds (Lau et al., 2007), but there have been no other detailed studies on the effect of AC in replanting cultivation. Moreover, little is known about the effect of RR amendment to the continuous replanting of asparagus. We investigated the effects of first and second and third planting and found that RR inhibits the growth of asparagus and growth was mostly inhibited after the third planting than the first and second and also found that P was the most inhibited nutrient in compared to other nutrient. Therefore to recover the growth and nutrient uptake after replanting, the purpose of the present investigation was to evaluate the growth of asparagus in subsequent cropping while investigating the impact of AC and P with a bias in the negative effect of RR amendment that would be beneficial in mitigating the continuous replanting problem.

2. Materials and Methods:

2.1 Planting material

UC157 crops have decreased in area owing to its reduced yield performance as compared to Gijnlim (Motoki et al., 2008). Therefore, to recover the growth, seeds of UC157 asparagus cultivar of USA origin, were obtained from a local commercial seed company (Sakata Seed Corporation, Japan).

2.2 Characteristics of the Sandy Soil

Physico-chemical properties of the soil are illustrated in **Table 1**. Sandy soil used in this experiment was first sterilized at 121°C for 15 min in an automatic high pressure steam

sterilized autoclave (MLS-2420; Sanyo, Tokyo, Japan). The soil was characterized as described in the chapter III.

2.3 Treatment conditions

The goals of this experiment were to investigate the effects of AC on plant growth, to know the effects of RR and AC, and to reveal the impacts of P on asparagus growth and nutrient uptake. Physico-chemical properties of the AC are illustrated in **Table 6**. The AC treatment included, by volume, 1.2 g powdered palm shell AC (Motoki et al., 2006) of the potting (size: 10X10 cm^2) soil and mixed homogeneously. The without AC treatment included, no addition (–) of AC. Fifteen years old asparagus roots were obtained from an asparagus field in Nagano prefecture, Japan. After air drying, they were separated by cutting them into approximately 1 cm lengths. The roots were then powdered with a rotary shaker and mixed into the soil at the rate of 2 g pot^{-1} (Blok and Bollen, 1993). The without RR treatment included, no addition (–) of RR. To check the role of P to asparagus growth, 100 ml of a P0 (0), P1 (7.5), P2 (15.5) and P3 (22.5) solution (mg l^{-1}) from KH_2PO_4 (Nuruzzaman et al., 2005) was applied weekly to each pot, in different combination of AC and RR amended or unamended soils, with the following combinational patterns: P0AC+RR+; P0AC+RR–; P0AC–RR+; P1AC+RR+; P1AC+RR–; P1AC–RR+; P2AC+RR+; P2AC+RR–; P2AC–RR+; P3AC+RR+; P3AC+RR– ; P3AC–RR+. This experiment treatment was replicated three times.

2.4 Replant culture

Table 6 Characteristics of palm sell activated carbon

Activated Carbon	Palm shell
Raw and processed materials	Coconut shell
pH	9.9
Specific surface area	956 m^2 g^{-1}
Total pore volume	0.46 cc g^{-1}
Micro-pore surface area	867 m^2 g^{-1}
Micro-pore volume	0.35 cc g^{-1}
Meso-pore surface area	83 m^2 g^{-1}
Meso-pore volume	0.09 cc g^{-1}
Meso+Macro-pore surface area	89 m^2 g^{-1}
Pore diameter	0.7 nm
Iodine absorption performance	1050 mg g^{-1}
Methylene blue adsorption performance	180 mg g^{-1}

16 meshes are equal to 1 mm size by Test methods (Azinomoto Fine -Techno Co., Inc., Japan) for activated carbon in JAS K 1474-1991.

Prior to sowing, asparagus seeds were covered with a double layer of gauze and surface-sterilized in 70% ethanol and then rinsed in deionized water several times. The pre-germinated seedlings of the asparagus cultivar were grown in commercially available plastic black pot (size: 10X10 cm^2) in sand culture for the first times of planting (**Fig. 15**). They were incubated at: 25° C; 12h light/12h dark; relative humidity 80% and 200 µmol m^{-2} s^{-1} intensity of light in growth chamber (MLR-351H; Sanyo, Tokyo, Japan) for 56 days (**Fig. 16**). Each pot was irrigated with distilled water according to field capacity. Field capacity was calculated as (wet mass- dry mass)/ dry mass X 100% (Lambers et al., 2002). Nutrients were provided with 10-20 ml Hoagland's P free nutrient solution everyday to avoid immobilization of nutrients. The seedlings were then harvested after 56 days and new seeds were replanted again in the same pot by using the same soil for the second (for 56 days) and third plantings (for 56 days), respectively by following the same process as above. In previous study we reveled the growth inhibition was highest in the third planting after the three subsequent replanting. Therefore, to recover the growth inhibition at maximum inhibitory stage data shown after the third planting stage. All treatments were replicated three times. Inhibition, expressed as a percentage, was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - Xt/Xc) \times 100 \qquad (1)$$

Where, Xc denotes the dry mass of the roots or shoots of the control and Xt represents the mean values of the corresponding dry mass of the treatments.

2.5 Plant growth and nutrient uptake measurements

After harvesting, all seedlings were carefully separated into roots and shoots, thoroughly cleaned, blotted dry between absorbing paper and their dry mass (g pot^{-1}) were measured after oven drying at 70°C for 72h. All dry roots and shoots were combined and ground to a

Fig. 15 Asparagus replanting with activated carbon (AC), phosphorus (P) and root residue amendment (RR)

fine powder using a stainless ball mill, and analyzed for total nutrients (mg g^{-1}) N, P and K using standard procedures. Total N content was determined by the dry combustion method with an automated C-N coder (Model MT 700; Yonaco, Tokyo, Japan). Total K content was determined after digestion with a H_2O_2-H_2SO_4 mixture. Total P in the digested mixture was determined colorimetrically with a spectrophotometer (Model U-2001, Hitachi Co., Tokyo, Japan) using the phosphomolybdate blue method (Murphy and Riley, 1962). Total K content was determined with an atomic absorption spectrophotometer (Model Z-8100, polarized Zeeman; Hitachi Co., Tokyo, Japan). All samples were run in triplicate. Inhibition, expressed as a percentage, for nutrient uptake was calculated by using equation 1.

2.6 Chemical analysis of soil after subsequent replanting

AC, RR and different levels of P amended or unamended soils were analysis for pH, electrical conductivity (EC) and C: N ratio. The pH and EC C: N was determined as described in the chapter III. Total mineral nutrients N, P and K were measured as described above. All treatments were replicated three times.

2.7 Statistical analysis

Experimental data presented are the means of three replicates; Statistical analyses were executed using Stat View software. The percentage data was $\log_e$-transformed before analysis where necessary to equalize variances between treatments (Lam et al. 2012). Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means. In addition, principle component analysis (PCA) performed to elucidate total data variability with respect to co-relationships for growth, nutrient uptake, and physicochemical characteristics. Moreover, each combination was treated as categorical data, converted to dummy variables and PCA performed along with other variables. Due to very low cumulative contribution ratio, some dummy variables were omitted.

Fig. 16 Asparagus replanting under laboratory condition with AC, P and RR

3. Results and discussion

3.1 AC and P reducing the negative impact of RR on growth

When asparagus was grown in soils that had been or had not been amended with AC and RR with different levels of P, responded differentially to asparagus growth while, RR tended to decrease the growth of the allelopathic potential asparagus after the third planting (**Fig. 17 a** and **b**). Root (**Fig. 17 a**) and shoot (**Fig. 17 b**) growth inhibition were significantly ($P < 0.05$) higher in P0AC–RR+combination than in P3AC+RR–. Root growth was more severely inhibited than shoot growth, since the root was more closely in contacted with the growing medium. On average, the application of AC increased asparagus growth with the increased level of P. The increase in the level of P concentration supply increased asparagus growth even it had been amended with or without AC and RR. Notably, after the third planting for which detected the effects between AC and RR showed that the inhibitory effect of RR in the presence of AC could be reduced as a result of the positive effect of AC on biomass of root and shoot because RR also tended to respond negatively to AC. This result indicated that RR could be responsible for growth reduction by producing toxic compounds. Shilling et al. (1992) examined that AC (0, 6, 12 v/v) partially reversed the inhibitory effect of celery residue (0.5, 1.0, 2.0 v/v) on lettuce growth inhibition were 12, 16, 0, 30, 39, 35, 40, 44 and 41 %, respectively in different combination was occurred instead of inhibition presumably by adsorbing phytotoxic compounds

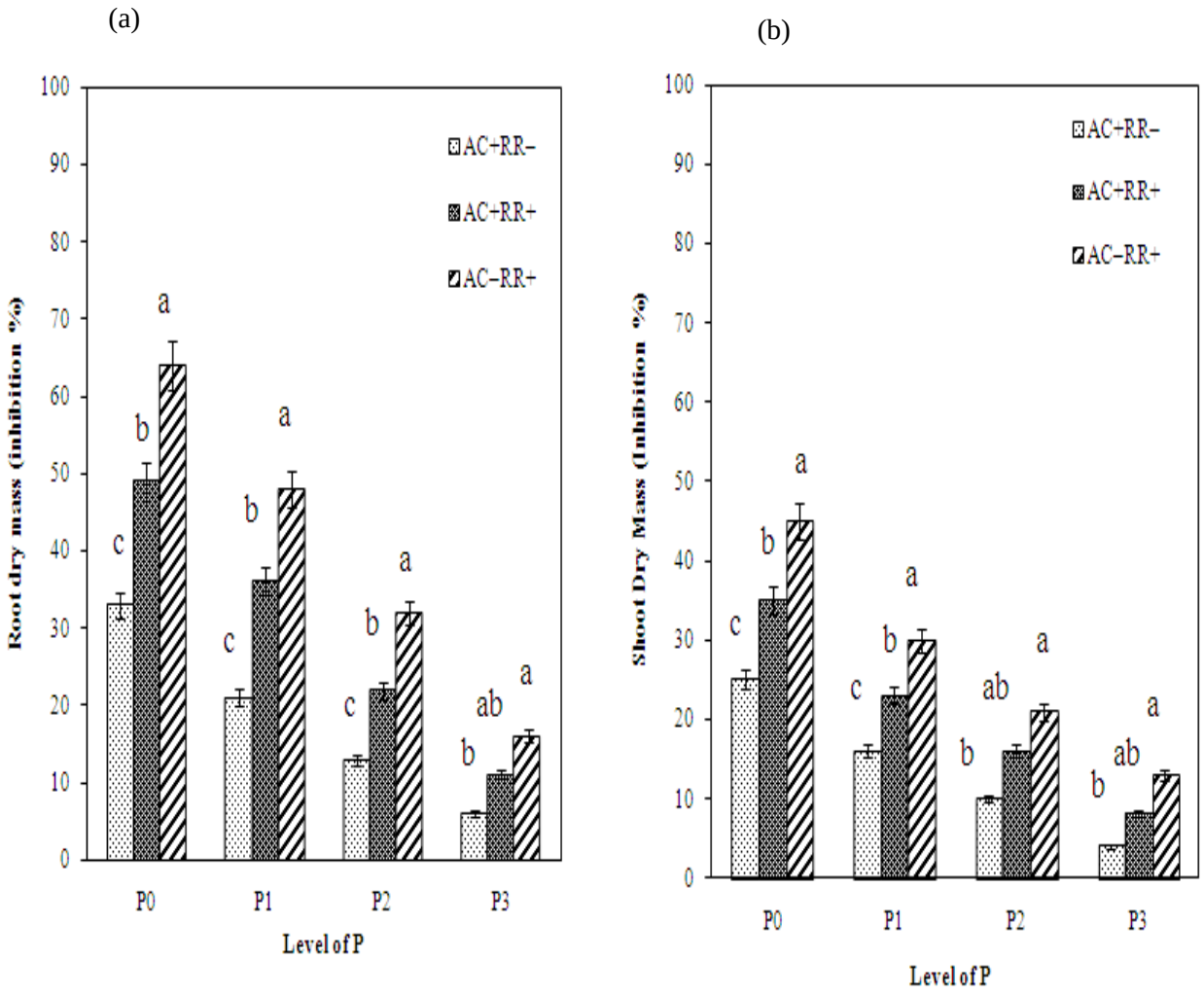


Fig. 17 Percent inhibition of asparagus seedling roots (a) and shoots (b) growth after the third replanting.

Note: Listed growth parameter suggests the values are actual dry mass (g pot^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

produced by celery, but total reversal was absent, probably because the AC did not adsorb 100% of the toxic substances. In this study, the mixture of AC with the different concentration of P led to the production of asparagus seedlings with a high growth while reducing the effects of RR amendment. Whatever the underlying mechanism(s), it was found that positive responses of combined AC and P influenced plant growth in the absence of the RR means that the biomass can be greater with AC and P than without AC and P.

3.2 Effect on nutrient uptake

The reduction for total nutrient uptake; N, P, K, were ranged from 5-38; 10-57; 6-26 %, respectively, after the third planting (**Fig. 18 a, b** and **c**). In this study, P (**Fig. 18 b**) was the most inhibited nutrient among N (**Fig. 18 a**) and K (**Fig. 18 c**). The highest decreased ($P < 0.05$) was found for P uptake (57%) in P0AC–RR+ combination, compared to P3AC+RR– combination, followed by N, while K varied with different combinations with and without RR, AC and P (0, 1, 2, 3). Asparagus nutrient uptake with RR amendment treatment showed the significant ($P < 0.05$) higher reduction in nutrient uptake in compared to RR unamendment treatment. This nutrient decreased also could be due to a release of phytotoxic substances by the interaction between RR and microorganisms. AC also may reduce microbial activity by reducing concentrations of organic molecules (Kulmatiski and Beard, 2006). Inderjit and Callaway (2003) recommend fertilizing pots to minimize the effects of trace concentrations of nutrients contributed by AC. Since, the RR could enhance the microbial biomass that may be responsible for subsequent reduction of nutrient. However, in AC unamendment treatment, detect the effects of RR for total nutrient content; RR also affects the P uptake and the other nutrient content of the plants.

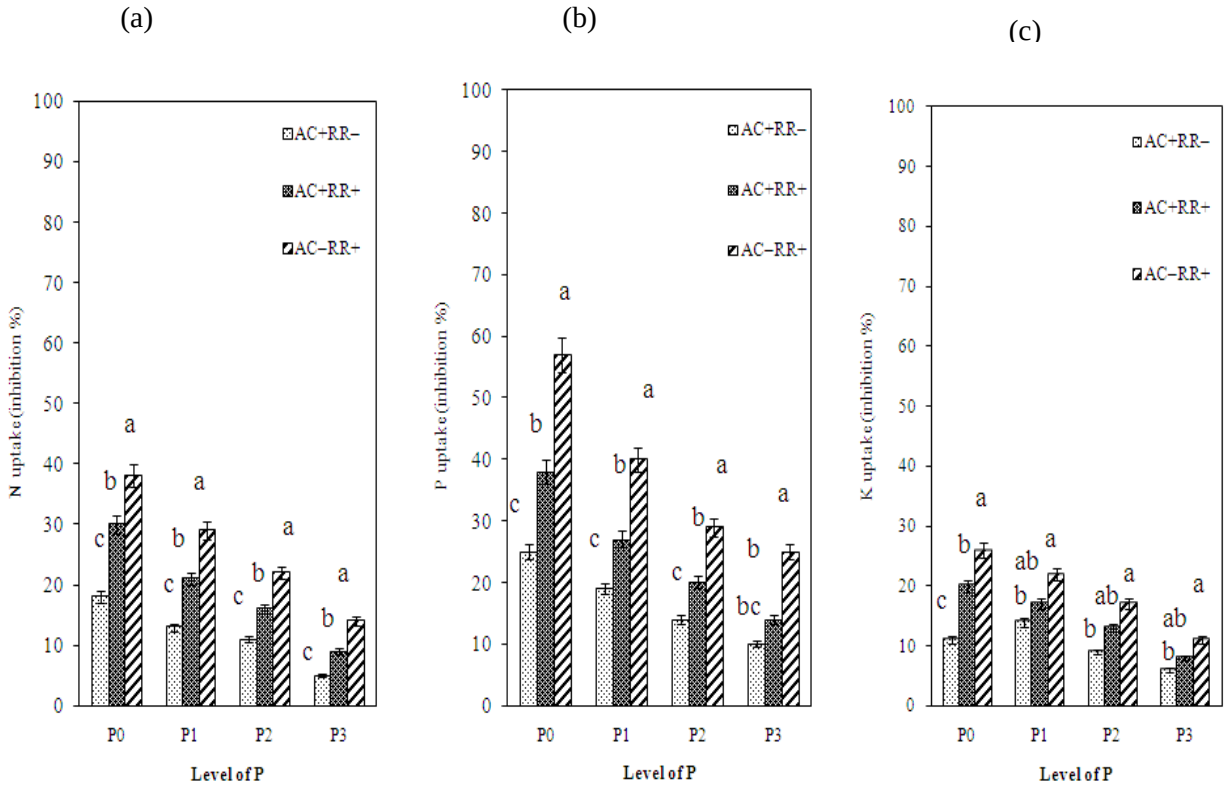


Fig. 18 Percent of inhibition of asparagus seedling nutrient uptake for N (a), P (b), K (c) after the third replanting.

Note: Listed nutrient suggests the values are actual concentration (mg g^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

RR trended for the highest reduction for P uptake among the other nutrient depending on the different combinations with AC and P0, P1, P2 and P3.

3.3 Effect of subsequent replanting on soil pH, EC and C: N ratio and nutrient uptake

Physico-chemical properties of the replanted soil were determined and the soil pH, EC and C: N ratio differed in with and between P3AC+RR– and P0AC–RR+ combination as a result of three subsequent replanting (**Fig. 19 a, b** and **c)**. The soil contained AC significantly ($P < 0.05$) increased the soil pH and C: N ratio and decreased soil EC, but the magnitude of that increase depended upon the RR amendment. The influence of pH, EC and C: N ratio, to the soil properties revealed that application AC and P is an important factor for the soil property. With the increasing of planting time the soil pH was decreasing and soil was becoming more acidic. On the contrary to, EC was increasing with the increasing of plating time. Increases in soil pH (Lucas and Davis, 1961), may result in increases in bio- available P, and to often pH-related increases in nutrient availability (Lehmann et al., 2003). This contrasting difference is reflected in the significant effect of C: N ratio. The soil contained AC increased the soil pH and C: N ratio but the magnitude of that increase depended upon the RR with different combinational treatments with P. The effect could be attributed to the high surface area of AC. After third planting, the reduction for total soil nutrient uptake; N, P and K were ranged from 9-48; 13-67; 8-36 %, respectively (**Fig. 19 d, e** and **f**). Soil P was the most inhibited nutrient among N and K. The highest decreased was found for P uptake (67%) in P0AC-RR+ combination, compared to P3AC+RR- combination. This result imply that the addition of AC and P to a sandy soil, making soil moisture and nutrients more available to plants growing to the soil, and eventually in improving crop productivity. Therefore, it is likely that allelochemical compounds could be readily leached from the residues.

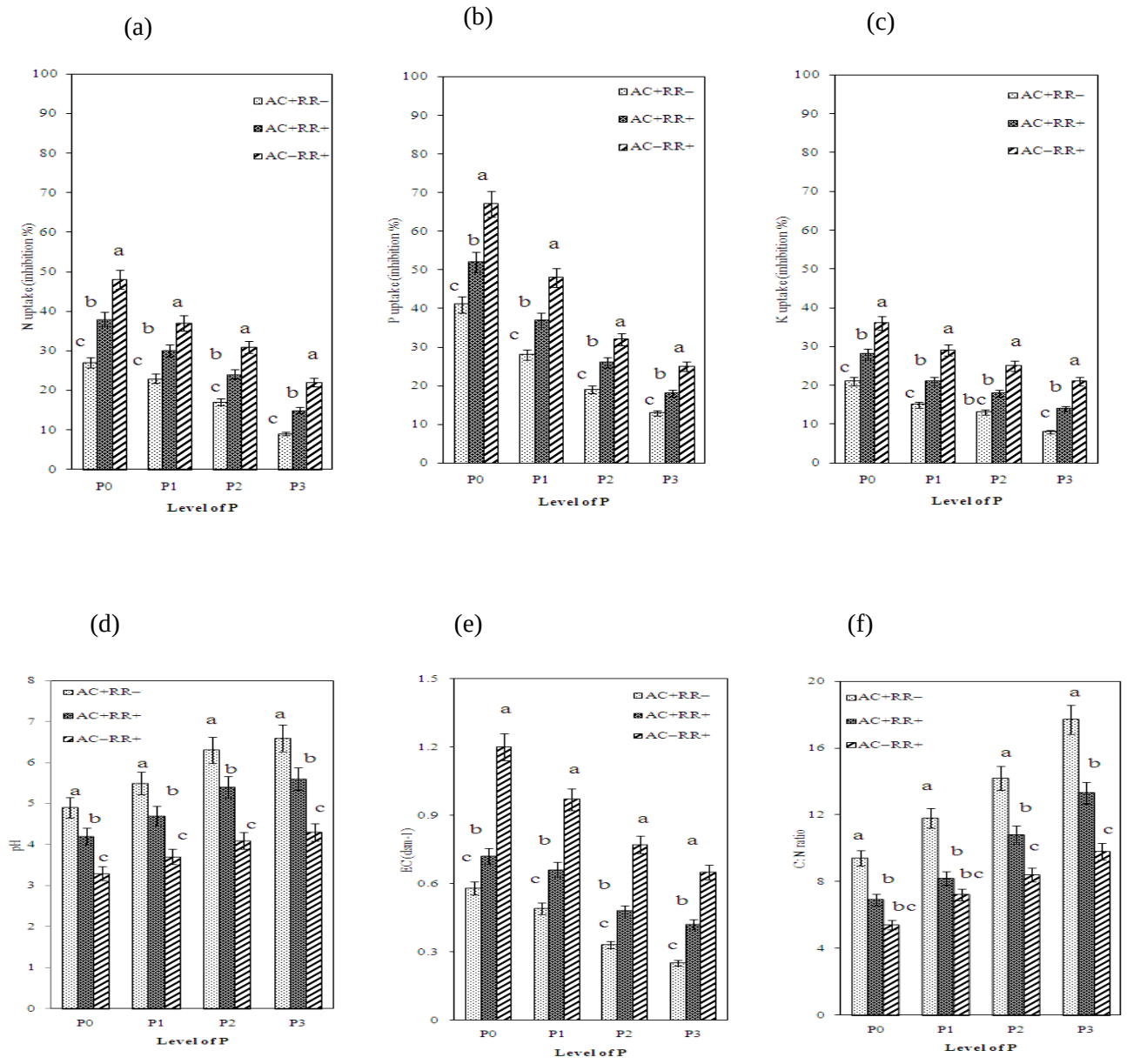


Fig. 19 Effects on soil physic-chemical properties to pH (a), EC (b), C: N ratio (c) after the third replanting. Percentage of inhibition of soil nutrient uptake for N (d), P (e) and K (f) after the third planting.

Note: Listed nutrient suggests the values are actual concentration (mg g^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

After entering into the soil, allelochemicals are influenced by microorganisms (Inderjit, 2001). However, the negative effect of allelochemicals depends greatly upon a variety of biotic and abiotic factors, soil type, presence of microorganisms and soil conditions, and further toxification and detoxification mechanisms in the soil (Blum et al., 1999).

3.4 Overall Physiochemical Characteristics variability

Principle component analysis (PCA) of the total variation among all the treatment with the growth and nutrient uptake (**Fig. 20 a**) and that among physiochemical characteristics (**Fig. 20 b**) were determined. There is strong positive co-relation in the combination P3AC+RR–, P2AC+RR–, P1AC+RR–, and P0AC+RR–, although negatively correlated with P3AC–RR+, P2AC–RR+, P1AC–RR+ and P0AC–RR+ combinations (**Fig. 20 a**). A positive co-relation between growth, nutrient uptake and soil properties by asparagus varieties were found after the third planting (**Fig. 20 b**). This reaffirms the significance of growth and nutrient uptake as earlier described. Among the physiochemical interactions, pH, N and P were strongly positively, but negatively correlated with EC and planting time. The strong positive correlation between N and P further suggests that nutrient uptake reduction might have been the precursor for the low pH and C: N ratio. Interestingly, the growth trends were opposite for when the soil contained AC and P, all significant effects of AC and P were found to be associated with increases in soil pH and C: N. In this study, AC and P significantly ($P < 0.05$) increased the growth of asparagus (**Fig. 17**). Also, AC was found to enhance nutrient uptake (**Fig. 18**), but to have only RR have negative effects on asparagus growth and nutrient uptake (**Fig. 17** and **18**) and soil properties (**Fig. 19**).

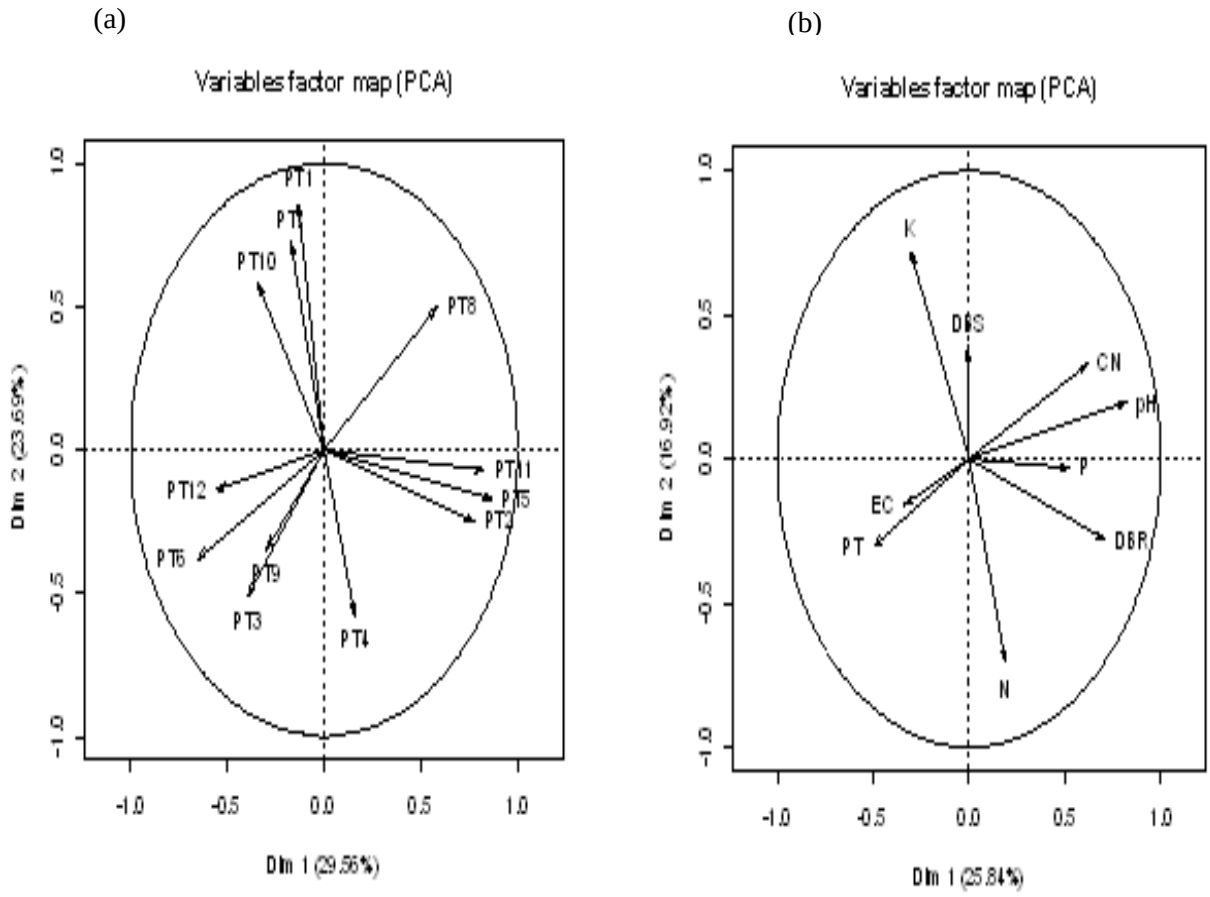


Fig. 20 (a) Variable factor map (PCA) for total variation after the three planting with different level of P, under AC and RR amendment and unamendment treatments. Where, P0AC+RR+ = PT1; P0AC+RR– = PT2; P0AC–RR+ = PT3; P1AC+RR+ = PT4; P1AC+RR– = PT5; P1AC–RR+ = PT6; P2AC+RR+ = PT7; P2AC+RR– = PT8; P2AC–RR+ = PT9; P3AC+RR+ = PT10; P3AC+RR– = PT11; P3AC–RR+ = PT12. Dim 1 and Dim 2 explain 29.56 and 23.69 % of the variation observed, respectively. **(b)** Variable factor map (PCA) for total variation in physiochemical characteristics with all the planting. Dim 1 and Dim 2 explain 25.84 and 16.92 % of the variation observed, respectively. Where, DBR = dry mass of root, DBS = dry mass of shoot, PT = planting time.

These provided the evidence that addition of AC with P might be very important in replanting system to maximize the growth and nutrient uptake. Since, the asparagus growth was inhibited due to continuous replanting (Blok and Bollen, 1993). The effect of previously grown plants on subsequent asparagus P content correlates well with growth of asparagus. These experiments show that additions of AC and P materials generally result in the alteration of physic-chemical properties that may lead to increases in nutrient uptake.

CHAPTER VI

Activated carbon and phosphorus application influences the growth of continuously replanted asparagus (*Asparagus officinalis* L.)

1. Introduction

Continuous cropping is a common phenomenon in asparagus (*Asparagus officinalis* L.) cultivation. After a few years with normal yields, crop growth is declined and consequently yield is gradually decreased. In previous chapter we found that root residue exudates of asparagus inhibited the roots and shoots growth of asparagus own seedlings, suggesting autotoxicity was a possible mechanism for the problem of declined yield and replanting problem. Moreover, we investigated the continuous cropping for asparagus production caused serious replanting problem, which inhibited mainly phosphorus (P) uptake in mineral nutrients. P is an essential element for higher plants and required in substantial concentration in plant tissues, particularly during vegetative growth. Suboptimal phosphorus supply diminishes. Much of the evidence in support of P as a key regulator of carbon partitioning has been obtained (Fredeen et al., 1989; Rychter and Randall, 1994). In terms of dry matter yield, the root is less affected than the shoot so that P deficient plants are typically low in shoot-to-root dry weight ratio (Fredeen et al., 1989; Heuwinkel et al., 1992). In general, under low P availability plant biomass accumulation decreases (Zobel et al., 2006), leaf growth is markedly reduced (Assuero et al., 2004) and root morphology is modified (Lambers et al., 2006). Under these conditions, an increases in root: shoot ratio has also been found, possibly due to a higher allocation of assimilates to roots (Vance et al., 2003; Hermans et al., 2006). Huge differences in plant responses to low soil P availability have been found among species and cultivars (Raghothama and Karthikeyan, 2005). The increase in P uptake can be achieved through architectural changes (e.g. increased root length, lower root diameter; higher density

and length of root hairs, enhanced lateral root formation, formation of cluster roots), functional changes (e.g. enrichment of high-affinity inorganic P transporters in the epidermis of roots and root hairs) and symbiosis with mycorrhiza that increase the exploration of the soil (Vance et al., 2003; Raghothama and Karthikeyan, 2005). However, reports on the replanting problem and appropriate variety selected are scant.

AC has been used to substantially reduce negative effects of plant–plant competition by adsorption of allelopathic substances excreted by plant roots (Kumar et al., 2009). Furthermore, AC additions would also be expected to increase the growth of plants (Kulmatiski and Beard, 2006). Moreover, little is known about the effect of RR amendment to the continuous replanting of asparagus. We investigated the effects of first and second and third planting and found that RR inhibits the growth of asparagus and growth was mostly inhibited after the third planting than the first and second and also found that P was the most inhibited nutrient in compared to other nutrient. This experiment was conducted to recover the asparagus growth after the first, second and third replanting by using Gijnlim cultivar for the investigating the impact of AC and P.

2. Materials and Methods:

2.1 Planting materials

A replant culture system was employed to identify the growth inhibitory activity of asparagus. Seeds of asparagus cultivar, Gijnlim of the European origin, were obtained from a local commercial seed company (Sakata Seed Corporation, Yokohama, Japan). Fifteen years old asparagus roots were obtained from an asparagus field in Nagano prefecture, Japan.

2.2 Characteristics of the Sandy Soil

Physico-chemical properties of the soil are illustrated in **Table 1**. Sandy soil used in this experiment was first sterilized at 121°C for 15 min in an automatic high pressure steam sterilized autoclave (MLS-2420; Sanyo, Tokyo, Japan). Prior to showing, the soil was characterized as described in the section VII.

2.3 Treatment conditions and replant culture

This experiment investigated the effects of AC on plant growth, to know the effects of RR and AC, and to reveal the impacts of P on asparagus growth and nutrient uptake. Physico-chemical properties of the AC are illustrated in **Table 6**. In this study the treatment condition was the same as described in the chapter VII. The treatment included, by volume, 1.2 g powdered palm shell AC (Motoki et al., 2006) of the potting (size: 10X10 cm^2) soil. The roots were then powdered with a rotary shaker and mixed into the soil at the rate of 2 g pot^{-1} (Blok and Bollen, 1993). They were incubated at: 25° C; 12h light/12h dark; relative humidity 80% and 200 µmol m^{-2} s^{-1} intensity of light in growth chamber (MLR-351H; Sanyo, Tokyo, Japan) for 56 days. To check the role of P to asparagus growth, 100 ml of a P0 (0), P1 (7.5), P2 (15.5) and P3 (22.5) solution (mg l^{-1}) from KH_2PO_4 (Nuruzzaman et al., 2005) was applied weekly to each pot, with the following combinational patterns: P0AC+RR+; P0AC+RR–; P0AC–RR+; P1AC+RR+; P1AC+RR–; P1AC–RR+; P2AC+RR+; P2AC+RR–; P2AC–RR+; P3AC+RR+; P3AC+RR– ; P3AC–RR+. The seedlings were then harvested after 56 days and new seeds were replanted again in the same pot by using the same soil for the second (first planting for 56 days) and third plantings (second replanting for 56 days) and forth plantings (third replanting for 56 days), respectively by following the same process as above. All treatments were replicated three times. Inhibition, expressed as a percentage, was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - Xt/Xc) \times 100 \qquad (1)$$

Where, Xc denotes the dry mass of the roots or shoots of the control and Xt represents the mean values of the corresponding dry mass of the treatments.

2.4 Plant growth and P uptake measurements

After harvesting, all seedlings were thoroughly cleaned; blotted dry between absorbing paper and their dry mass (g pot^{-1}) were measured after oven drying at 70°C for 72h. All dry roots and shoots were combined and ground to a fine powder using a stainless ball mill, and analyzed for total P (mg g^{-1}) using standard procedures. Total P in the digested mixture was determined colorimetrically with a spectrophotometer (Model U-2001, Hitachi Co., Tokyo, Japan) using the phosphomolybdate blue method (Murphy and Riley, 1962). All samples were run in triplicate.

Inhibition, expressed as a percentage, for P uptake was calculated by using equation 1.

2.5 Chemical analysis of soil after subsequent replanting

AC, RR and different levels of P amended or unamended soils pH, electrical conductivity (EC) and C: N ratio was determined by procedures already described in preceding sections. Total P was measured as described above. All treatments were replicated three times.

2.6 Statistical analysis

Experimental data presented are the means of three replicates; statistical analyses were executed using Stat View software. The percentage data was $\log_e$-transformed before analysis where necessary to equalize variances between treatments (Lam et al. 2012). Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means.

3. Results and discussion

3.1 Impact of AC and P on growth

AC and RR with different levels of P, responded differentially to asparagus growth after the three subsequent replanting (**Fig. 21 a, b** and **c**). After the first, second and third replanting, the highest (23, 35 and 55%) and lowest (2, 3 and 8%) inhibitions for growth of root were found in the P0AC–RR+ and P3AC+RR–, respectively. Application of AC could reduce allelopathic effects and increased plant growth and yield (Ridenour and Callaway, 2001). In this study, the combined application of AC and P led to the production of asparagus seedlings with a high growth while reducing the effects of RR amendment. The positive effect of AC on growth could be a result of AC minimizing any negative growth effects of allelopathic compounds.

3.2 Effect on P uptake

The highest inhibition ($P < 0.05$) was found for P uptake (52%) in P0AC–RR+ combination, compared to P3AC+RR– combination after the third replanting, followed by first and second replanting (**Fig. 22 a, b** and **c**). Asparagus P uptake with RR amendment treatment showed the significant ($P < 0.05$) higher reduction in P uptake in compared to RR unamendment treatment. This nutrient decreased also could be due to a release of phytotoxic substances by the interaction between RR and microorganisms. Since, the RR could enhance the microbial biomass that may be responsible for subsequent reduction of P uptake. However, in AC unamendment treatment, detect the effects of RR for total nutrient content; RR also affects the P uptake. RR trended for the highest reduction for P uptake depending on the different combinations with AC and P0, P1, P2 and P3. Thus, the combined application of AC and P could be useful for replenishment allelopathy. The evolutionary adaptations of plants to P deficiency include responses that help

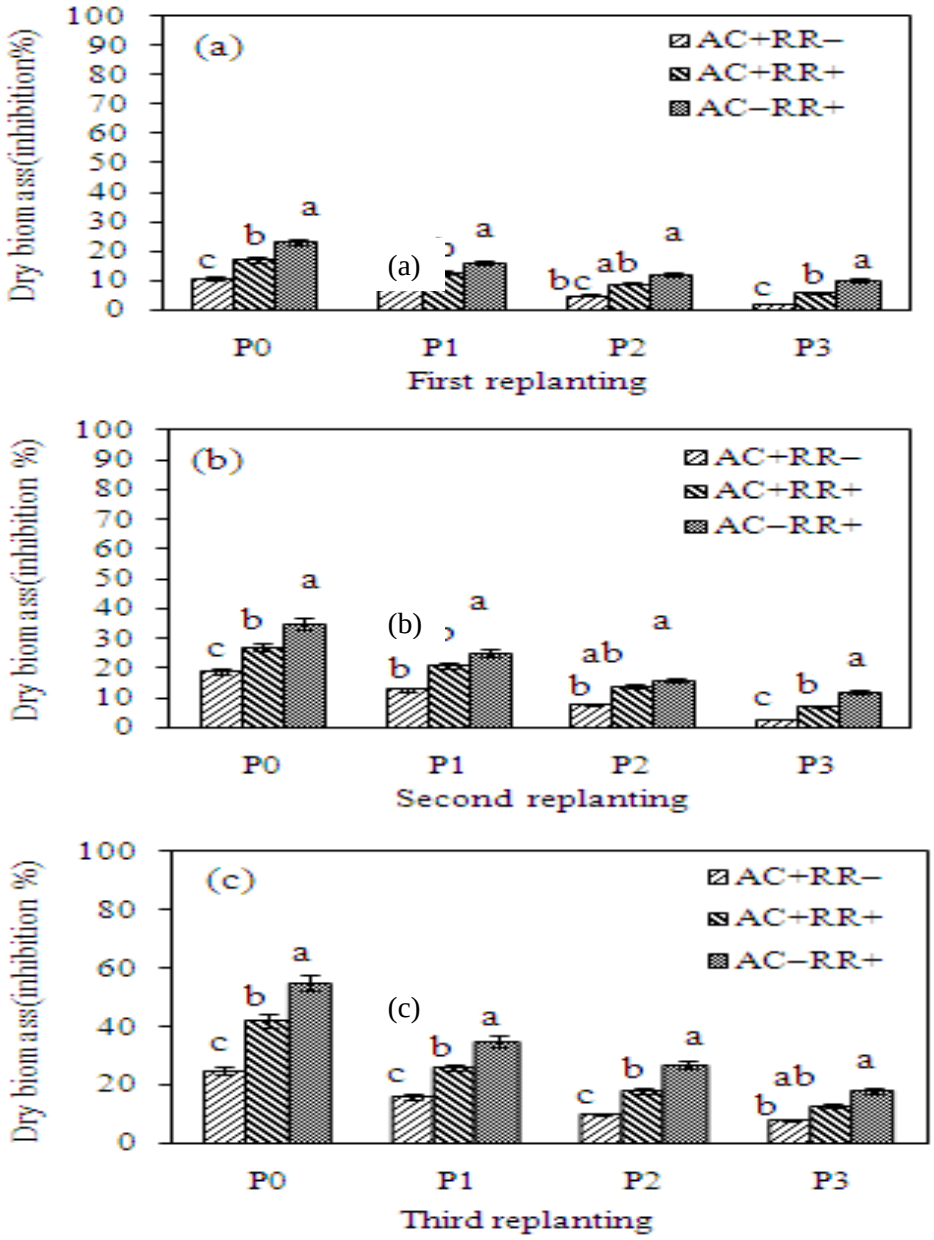


Fig. 21 Percent inhibition of asparagus seedling roots growth after the first (a), second (b) and third (c) replanting. Listed growth parameter suggests the values are actual dry mass (g· pot^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

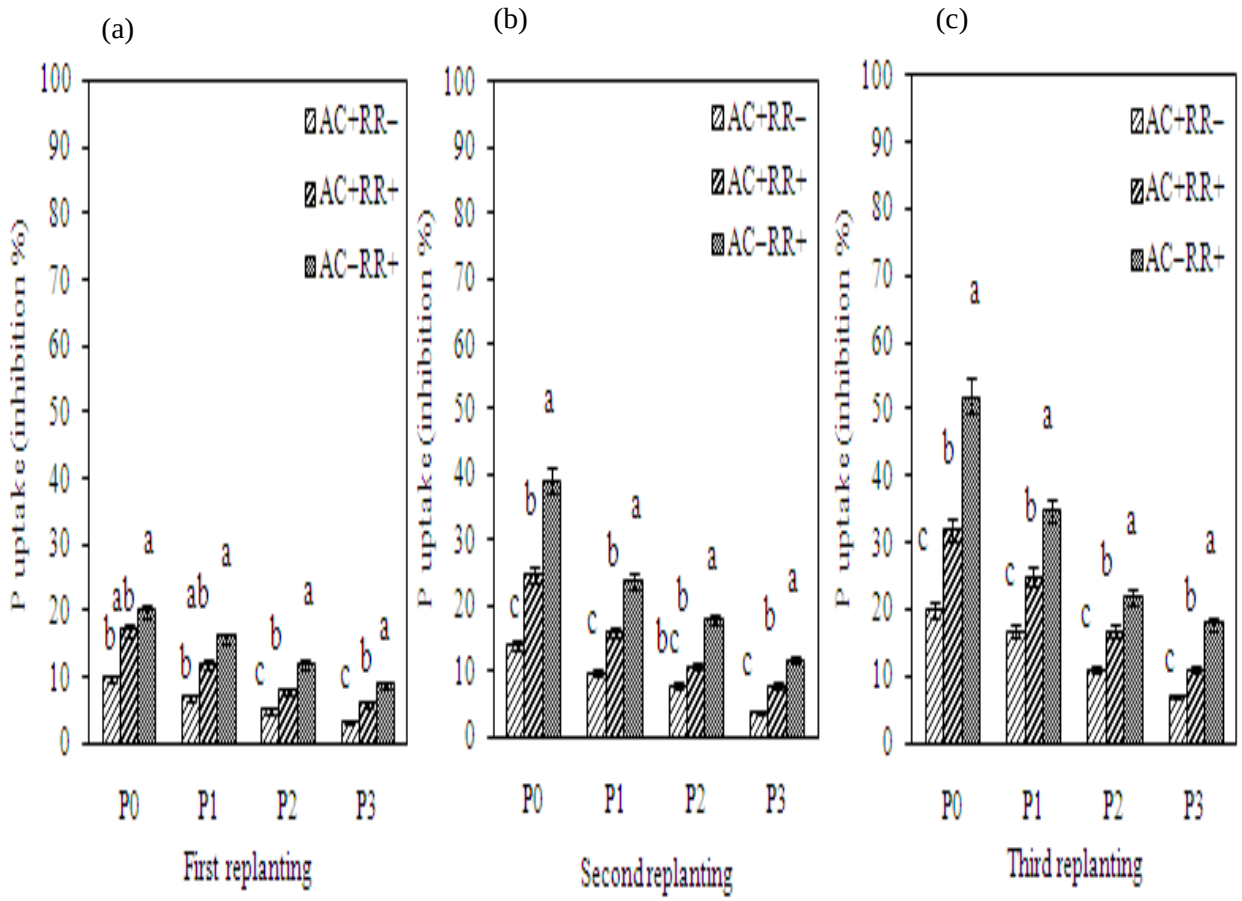


Fig. 22 Percent inhibition of asparagus seedling P uptake after the first (a), second (b) and third (c) replanting. Listed nutrient suggests the values are actual concentration (mg g^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

them enhance the soil P availability, increase its uptake and improve the use efficiency of P within a plant (Lambers et al., 2006). Plants have developed biochemical mechanisms to mobilize P in the rhizosphere (Lynch, 2007). However, our results show that AC and P influenced nutrient uptake in both the presence and the absence of RR. AC can increase nitrification, in part because AC may sorbs compounds that are inhibitory to nitrifying bacteria (Deluca et al., 2002, 2006). Therefore, AC and P can be a good tool for studies of allelopathy because it acts as an adsorbent for many large organic compounds and increased the nutrient uptake.

3.3 Effect of subsequent replanting on soil pH, EC and C: N ratio and P uptake

Physico-chemical properties of the replanted soil were determined and the soil pH, EC and C: N ratio differed in with and between P3AC+RR– and P0AC–RR+ combination as a result of three subsequent replanting (**Table 7**). The soil contained AC significantly ($P < 0.05$) increased the soil pH and C: N ratio and decreased soil EC. With the increasing of planting time the soil pH was decreasing and soil was becoming more acidic. But, EC was increasing with the increasing of plating time. This contrasting difference is reflected in the significant effect of C: N ratio. The soil contained AC increased the soil pH and C: N ratio but the magnitude of that increase depended upon the RR with different combinational treatments with P. The effect could be attributed to the high surface area of AC. The reduction for total soil P uptake ranged from 5-63 % (**Fig. 23 a, b** and **c**). After third replanting, the highest and lowest decreased was found for soil P uptake (12 and 63%) in P0AC-RR+ and P3AC+RR- combination, respectively. This result imply that the addition of AC and P to a sandy soil, making soil moisture and nutrients more available to plants growing to the soil, and eventually in improving crop productivity. Additionally, Lehmann et al. (2003) showed that AC itself contained small amount of nutrients that would be available to both soil biota and

plant roots. Based on the observed results, the present study evidences that root residues of *Asparagus officinalis* suppress the growth of asparagus by releasing allelochemicals into the soil rhizosphere through alteration of soil P which could be improve by the combined application of AC and P.

Table 7 Effects of phosphorus (P) and activated carbon (AC) application on soil physic-chemical properties after the first, second and third plantings.

	First replanting			Second replanting			Third replanting		
Treatment	AC+RR–	AC+RR+	AC–RR+	AC+RR–	AC+RR+	AC–RR+	AC+RR–	AC+RR+	AC–RR+
				pH (H_20)					
P0	5.9±0.04d	5.4±0.05d	4.8±0.11d	5.1±0.02d	4.7±0.09d	4.3±0.01c	4.7±0.04c	4.4±0.05d	4.0±0.23d
P1	6.1±0.07c	5.8±0.03c	5.3±0.08c	5.6±0.01c	5.2±0.07c	4.8±0.06b	5.0±0.03bc	4.7±0.01c	4.4±0.65c
P2	6.7±0.05b	6.3±0.01b	5.7±0.06b	6.0±0.04b	5.7±0.01b	5.5±0.02ab	5.3±0.09b	5.1±0.03b	4.9±0.71b
P3	7.3±0.01a	6.7±0.02a	6.2±0.03a	6.5±0.07a	6.3±0.02a	5.7±0.02a	5.6±0.05a	5.5±0.00a	5.1±0.15a
				C:N ratio					
P0	20.4±0.66d	17.9±0.11d	14.4±0.71d	16.8±1.29d	14.4±2.13d	11.5±0.93d	12.9±1.31d	9.7±2.61d	7.1±1.93d
P1	24.8±1.02c	22.2±0.87c	18.2±0.94c	20.8±1.43c	18.8±1.21c	13.2±1.22c	15.2±2.14c	11.1±1.03c	9.9±1.43c
P2	30.2±1.51b	24.8±0.25b	20.4±0.58b	23.2±2.81b	20.2±1.18b	16.8±0.81b	17.8±1.03b	15.4±3.03b	11.4±2.03b
P3	35.7±2.78a	29.3±0.19a	25.8±1.92a	27.7±1.55a	24.7±3.27a	19.3±0.17a	21.3±1.23a	17.5±1.03a	14.8±1.03a
				EC(ds·m^1)					
P0	0.45±0.03a	0.53±0.06a	0.89±0.11a	0.68±0.04a	0.95±0.01a	1.8±0.06a	1.3±0.02a	1.6±0.15a	2.0±0.01a
P1	0.37±0.05b	0.44±0.04b	0.84±0.09b	0.65±0.06b	0.92±0.09b	1.4±0.02b	0.91±0.04b	1.3±0.03b	1.8±0.05b
P2	0.24±0.01c	0.38±0.05c	0.72±0.05c	0.43±0.03c	0.78±0.05c	1.1±0.02c	0.83±0.09c	1.0±0.07c	1.6±0.17c
P3	0.18±0.07d	0.27±0.01d	0.63±0.02d	0.40±0.01d	0.66±0.02d	0.98±0.09d	0.75±0.01d	0.83±0.01d	1.2±0.08d

Lettering (a, b, c) in the table refers to comparisons for the first and second replantings separately (Tukey's protected multiple-comparison test, $p < 0.05$) and data presented as mean ± SE, replication ($n = 3$).

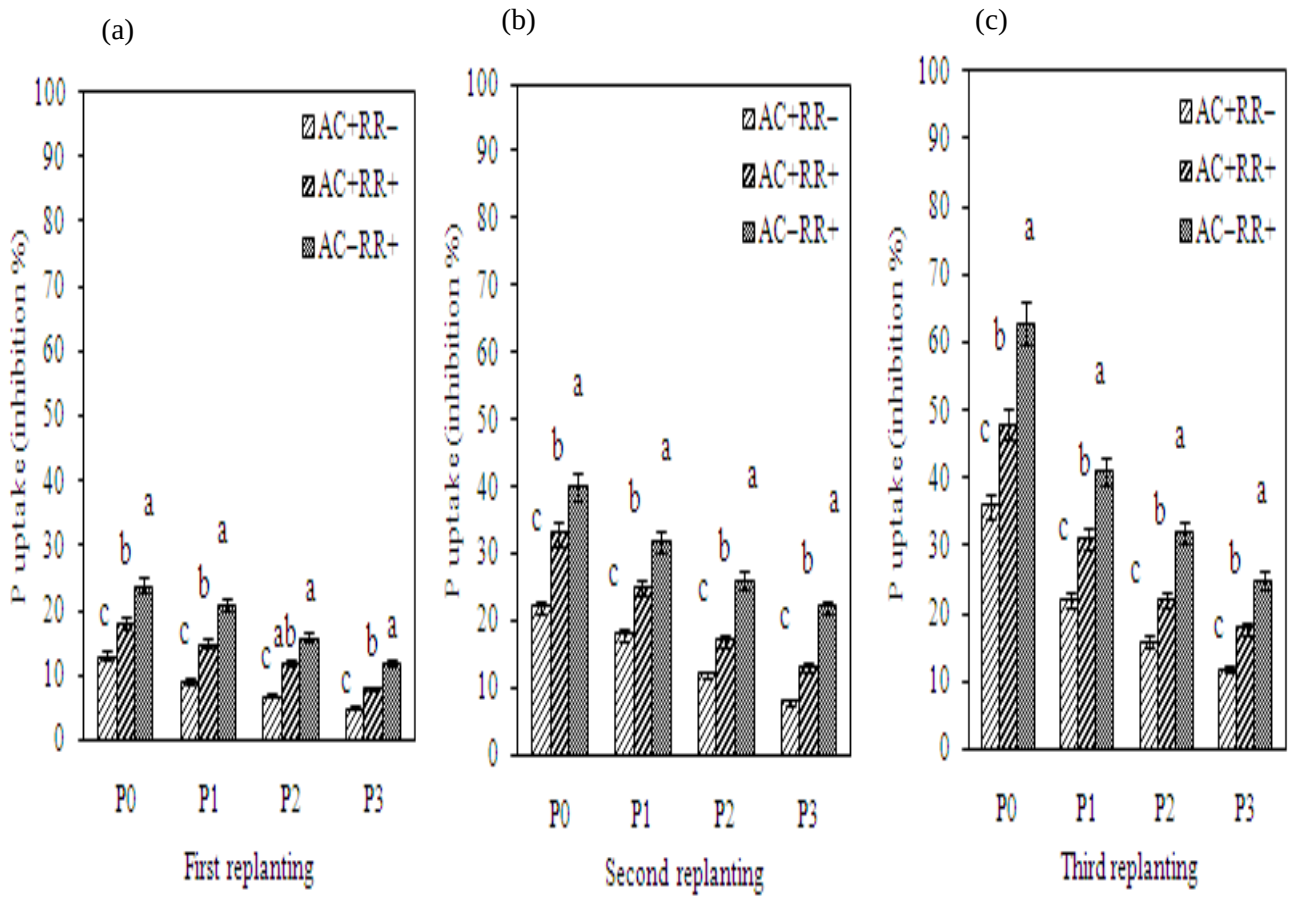


Fig. 23 Percent inhibition of soil P uptake after the first (a), second (b) and third (c) replanting. Listed nutrient suggests the values are actual concentration (mg g^{-1}). P0, P1, P2 and P3 denote 0, 7.5, 15.5 and 22.5 mg l^{-1} of applied P. Inhibition (%) was calculated by using equation 1. Bars presented as mean ± SE, Tukey's protected multiple-comparison test at $P < 0.05$ was used to compare means, replication (n= 3).

CHAPTER VII

SUMMARY AND CONCLUSIONS

Asparagus has a replanting problem regarding of allelopathy when retransplanted new seedlings at the same field. Therefore, we conducted three different experiments to clarify growth and nutrient uptake inhibition and how to recover under replanting conditions. In this study, we evaluated the allelopathic potential and autotoxic interference with growth and nutrient uptake of two asparagus varieties; UC157 (U) and Gijnlim (G) from USA and Europe, respectively. The two varieties were cultivated in a continuous replanting system in different rotational patterns with soil amended by asparagus root residues, and unamended soil (control) under greenhouse conditions. The combinations consisted of: G, U and GU, UG, GG, UU and GUG, GUU, GGU, UGU, UUG, UGG, GGG, UUU for the first planting, first replanting and second replanting, respectively. In this study, UC157 showed higher varietal allelopathic and autotoxic effects as compared to Gijnlim. Root and shoot growth were significantly ($P \leq 0.05$) inhibited by up to 83 and 91 %, respectively in the second replanting of UC157 (UUU) when grown in the root residues amended soil; suggesting that root residues were responsible for the replanting problem. Further, growth inhibition was correlated with nutrient uptake inhibition; phosphorus (P) uptake was the most inhibited nutrient among nitrogen (N), potassium (K), calcium (Ca), and magnesium (Mg). Contrastingly, UC157 altered soil composition significantly ($P \leq 0.05$) more than Gijnlim after subsequent replanting; for instance pH, C: N ratio and N, P, K, Ca and Mg were decreased whereas electrical conductivity (EC) was increased. Principle component analysis (PCA) of the total variation in different rotational combinations in the inhibitory effect of root residue amendment after the first, second replanting showed the correlation ship within the same replanting treatments among different rotational combination.

Allelopathy research in various aspects has been applied to the fields of agriculture and forestry in order to reduce environmental pollution and increase agricultural production for sustainable agriculture. Allelochemicals are a major reason for replanting problem of asparagus. The effects of potential allelochemicals on the growth of asparagus cultivars UC157 and Gijnlim in replant culture were investigated. Their contents exuded from the roots of each cultivar were determined by HPLC, and their phytotoxicity during the replanting time assessed in agar medium. Organic acids, oxalic, succinic and tartaric, were the main identified allelochemical compounds from the roots of asparagus cultivars. The second replanting produced a higher concentration of total allelochemicals than the first. In general, root and shoot biomass were inhibited by up to 81 and 68%, respectively in the second replanting of UC157. The inhibition of lettuce growth by oxalic, succinic and tartaric acids was also investigated, using a bioassay. The inhibitory concentration (IC_{50}) and the effects of these allelochemicals compounds, oxalic (11 mg l^{-1}), succinic (18 mg l^{-1}) and tartaric (6 mg l^{-1}) acids might have an important function in the allelopathic responses of asparagus replanting problem.

The influence of varietal resistance to allelopathy and autotoxicity for growth, nutrient uptake and allelochemical characteristics were also assessed under laboratory conditions. The two asparagus varieties; UC157 (U) and Gijnlim (G) were cultivated in different rotational patterns in a continuous replanting system. The rotational combinations consisted of: UG, GU, GG, UU and GUG, GGU, GUU, UGU, UUG, UGG, GGG, UUU for the first and second replantings, respectively. The control planting was the first planting of each variety. The two varieties exhibited significant ($P< 0.05$) differences in growth, nutrient uptake and allelochemical characteristics while UC157 showed more varietal allelopathic and autotoxic activities than Gijnlim after two subsequent replantings. Root and shoot growth were inhibited by up to 77 and 73 %, respectively in the second replanting of UC157 (UUU) in

compared to control (first planting of UC157). Growth inhibition was correlated with nutrient uptake inhibition; phosphorus (P) uptake was the most inhibited nutrient among the other nutrients such as nitrogen (N), potassium (K), calcium (Ca), and magnesium (Mg). The identified allelochemicals were oxalic, succinic and tartaric acids in the root exudates of two varieties; whereas UC157 produced the highest concentration of total allelochemicals than Gijnlim; suggesting that allelochemicals could be responsible for both growth and nutrient uptake inhibitions in asparagus cultivation systems. Contrastingly, these inhibitions indicated that selection of suitable asparagus varieties and varietal rotations are necessary in replantings in order to minimize the negative impacts of varietal allelopathy and autotoxicity. The varying degree of inhibition with differential responses to the allelopathic and autotoxic compatibility may be valuable in predicting the potential growth and nutrient uptake inhibitions of subsequent asparagus cultivation. Therefore, we explored the causes of these problems and how to improve growth and nutrient uptake especially for phosphorus (P) uptake under replanting conditions with asparagus varieties. In addition, activated carbon (AC) is widely used technique for neutralizing allelopathic compounds which is secreted or decomposed from root residue (RR). However, this technique also directs effects on plants and soil because it alters growth, nutrient availability, pH and C: N ratio. This study investigated the effect of AC with RR incorporation and P on asparagus growth, nutrient uptake under controlled environmental conditions. AC was incorporated into sandy soil and was amended with 15 years old RR of UC157 before the sowing of asparagus for the first, second and third time of continuous planting. In all the planting, P was applied at P0 (0), P1 (7.5), P2 (15.5) and P3 (22.5) mg l^{-1}, asparagus seedlings growth and nutrient uptake was measured and data shown after third planting stage, caused maximum growth and nutrient uptake reduction. In the absence of RR, AC significantly increased plant growth and nutrient uptake, but when RR was incorporated decreased plant growth and nutrient uptake in all three

planting. When AC was incorporated into the soil as phosphorus (P3) increased level and without RR, asparagus root (80 %) and shoot (84 %) biomass increased, total nitrogen (N), phosphorus (P), potassium (K), uptake by 87, 82 and 76 %, respectively. The increased ($P < 0.05$) growth corresponded to increased plant P content, likely resulting from greater P availability. The difference between plant growth in medium with and without activated carbon and in the presence of the potentially allelopathic RR; however, this difference may be biased if AC with applied P alters soil nutrient availability and plant growth even in the presence of the RR allelopathic agent. RR incorporation, however, retarded the effects of fertilization on asparagus growth and P uptake. The combined application of AC and P increased asparagus growth and nutrient uptake. In this study, plant growth and P uptake was increased with the increasing of level of P, but it was not known the optimum level of P, until which level growth will be retarded, therefore, the present steps are currently validating to explore the exact quantity or level of P and to find out the mechanism and specific causes of these problems and how to improve the growth and P uptake under continuous replanting.

In conclusion, these studies provided additional evidence of asparagus varietal allelopathy and autotoxicity in different rotational combinations under greenhouse and laboratory conditions. The growth inhibition of asparagus could be mitigated by proper selection of varieties to reduce the persistence of autotoxins and accumulation of allelochemicals from root residues during replanting. AC and P application will be required under replanted soil to replenish the allelopathic effects of RR incorporation, or to improve growth and nutrient uptake, by overcoming nutrient immobilization resulting from RR amendment in asparagus replanting.

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Rumana Yeasmin, Ken Nakamatsu, Satoru Motoki, Hiroshi Matsumoto, Sadahiro Yamamoto, Eiji Nishihara. Inference of allelopathy and autotoxicity to varietal resistance of asparagus (*Asparagus officinalis* L.). Australian Journal of Crop Science (accepted, August, 2013)

This paper partly covers **Chapter IV** in the thesis.

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This paper partly covers **Chapter VI** in the thesis.

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アスパラガスの連作障害におけるアレロパシーおよび自家中毒の軽減に関する研究

アスパラガス(*Asparagus officinalis* L.)は栽培期間が比較的長く 15 年以上と言われている多年草の園芸作物の 1 つである．しかしアスパラガスは，同じほ場に新しい苗を改植すると原因不明の連作障害が発生し枯死するとった問題が発生している．その原因の 1 つにアレロパシーが関与していると考えられているが．なお不明な点が多い．そこで，改植時のアスパラガスのアレロパシー現象を根から滲出する物質を調査し，さらにどのような作付け体系を行なえば，アスパラガスの改植におけるアレロパシーを回避できるか品種の組み合わせおよび資材による改善効果について検討した．アスパラガス品種 UC157(U)，Gijnlim (G)の根から滲出したアレロパシー物質を調査した結果，有機酸の 1 種であるシュウ酸，コハク酸および酒石酸化合物が確認された．さらに，それらの物質の 50%阻害濃度(IC50)値をバイオアッセイ法で調査した結果，同定されたそれぞれの物質の濃度は 11 mg l-1，18 mg l-1 および 6 mg l-1 であり，それらの有機酸はアスパラガスの改植時におけるアスパラガスの成長にアレロパシー作用の上である程度影響を及ぼしていることが示唆された．次に、改植条件下におけるアスパラガスの成長，栄養吸収阻害およびそれらを改善させる方法を明らかにするためにビニルハウス(砂質土壌)および人工気象室(寒天培地)において 2 つのアスパラガス品種の連作を品種の異なる輪作の組み合わせにより実行した．2 つの品種のアスパラガス；UC157(U)，Gijnlim (G)はビニルハウスにて異なる輪作の組み合わせとアスパラガス根部残渣が添加された処理区とされていない処理区(対照区)における連作系において栽培された．輪作の組み合わせは以下の通りである：1 作目 G，U，改植 1 回目 GU，UG，GG および UU，改植 2 回目 GUG，GGU，GUU，UGU，UUG，UGG，GGG および UUU．その結果，根部残渣が加えられた処理区で栽培された改植 2 回目の UC157 (UUU)の根部とシュートの成長は対照区と比べビニルハウスではそれぞれ 83，93%，人工気象室ではそれぞれ 77，73%阻害された($P < 0.05$)．従って，UC157 は品種間におけるアレロパシー作用および自家中毒（同じ品種の連作）が Gijnlim と比べ高い水準であることが考えられた．また，このことから 1 作目の根部残渣が改植時における問題の原因であることが示唆された．更に，品種の組み合わせによる連作栽培には、成長阻害と栄養吸収阻害の間に相関関係が認められた．特にリン酸(P)の吸収は窒素(N)，カリウム(K)，カルシウム(Ca)およびマグネシウム(Mg)と比べ最も阻害された．ビニルハウスの試験では，UC157 が改植された後の土壌は Gijnlim と比べ有意に改変された($P \leq 0.05$)．即ち，C/N 比，N，P，K，Ca および Mg 濃度が減少し，電気伝導度(EC)が増加する傾向を示した．ビニルハウスの試験の全項目における根部残渣が添加された土壌による阻害を変数として主成分分析(PCA)を行った結果，改植 1，2 回目のそれぞれの輪作組み合わせ間に相互関係が認められた．また，アスパラガス品種のアレロパシー作用および自家中毒への

反応差による成長あるいは栄養吸収阻害の程度の差の把握は，その後のアスパラガス栽培の潜在的な成長と栄養吸収の阻害を予測する上で重要と考えられた．そこで，それらの阻害が発生する原因の究明とアスパラガス品種による改植条件下において成長および栄養吸収，特にリン酸の吸収の改善を，アスパラガスの連作障害を改善させることが知られている活性炭を用いて試みた．活性炭の施用はアスパラガス根部の残渣から生じた他感作用物質の吸着させる技術として広く用いられているが，活性炭の施用は土壌 pH および C/N 比をも改変させることによってアスパラガスの成長および栄養吸収に影響を与える可能性がある．そこで，人工気象室にて土壌への活性炭およびアスパラガス根部残渣の混合がアスパラガスのリン酸吸収に及ぼす影響を調査した．砂質土壌(対照区)とアスパラガス品種 UC157 が 15 年栽培されて根部の残渣も含まれている土壌に活性炭を混合し，アスパラガスを 3 回連続で連作した．改植に際して P を土壌に添加し，処理区を以下のように設定した；P0 (0 mg l−1), P1 (7.5 mg l−1)，P2 (15.5 mg l−1)および P3 (22.5 mg l−1)．その結果，P3 を連作土壌に施用することによってアスパラガスの根部およびシュートの乾燥重量が増加し，それぞれ対照区の 80，84%まで回復した．また，窒素，リン酸およびカリウムの吸収量はそれぞれ対照区の 87，82 および 76%まで回復した．乾燥重量の増加は植物体中のリン濃度の上昇と相関関係にあり，このことからアスパラガスのリン酸の利用性の増加が示唆された．しかし，活性炭が添加されておらず，根部残渣の存在下におけるアスパラガスの成長の違いは，活性炭とともに添加されたリン酸の土壌における利用性に変化が生じた場合，偏りが発生する可能性が考えられた．また，根部残渣の混合された土壌におけるアスパラガスの栄養吸収は低下するが，活性炭とリンの添加により改善されることが明らかとなった．また，リンの施用濃度の増加に伴いアスパラガスのリン酸吸収が増加することが明らかとなったが，連作土壌への最適なリン酸の濃度は依然として不明である．従って，今後はアスパラガスの成長に最適なリン酸濃度を明らかにすることがアスパラガスの連作障害の軽減の為に重要となる．以上のことから、アスパラガスの改植に対する新しいアスパラガスの成育不良には、多かれ少なかれアレロパシー物質が関与していることが明らかとなった．また，改植時におけるアスパラガスの品種に選定は，その後の改植時のアスパラガスの成育におけるアレロパシーの耐性に関与していることが明らかとなり，アレロパシー作用に対しての活性炭およびリン酸資材の単独あるいは組み合わせの投与は，アスパラガスの改植時のアスパラガスの成育を改善させる効果を明らかにした．今後はアスパラガスの改植時におけるアスパラガスの作付け対策のマニュアルの基礎データとなると考えられる．