Research on allelopathy of Vietnamese tea (*Camellia sinensis* (L.) Kuntze) and demonstration of caffeine as putative allelochemical in action

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Research on allelopathy of Vietnamese tea (*Camellia sinensis* (L.) Kuntze) and demonstration of caffeine as putative allelochemical in action

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This dissertation attached hitherto, entitled "Research on allelopathy of Vietnamese tea (*Camellia sinensis* (L.) Kuntze) and demonstration of caffeine as putative allelochemical in action" is being submitted as a doctoral dissertation in partial fulfilment of the requirement for the degree of Doctor of Philosophy (Ph.D. in Agricultural Sciences) by the United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology.

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DECLARATION OF ORIGINALITY

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

Pham Thi Thanh Van

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Abstract

Herbicides have become indispensable for crop protection from damage of various weeds, weed management has been mainly depending on synthetic herbicides. Widespread use of synthetic herbicides has resulted in herbicide-resistant weeds and the unsafe of it. Reducing the contamination of those herbicide to the environment is important step toward sustainable agriculture. In recent years, allelopathic species and allelochemicals have been applied as an alternative weed management method. Addition, the increasing possibilities for developing bio-herbicides from plants that could be used for weed control to minimize the heavy reliance on synthetic herbicides. Tea (*Camellia sinensis* (L.) Kuntze) is one of the most abundant plants in Vietnam by many functions in food and beverage. Many previous reports showed that caffeine and catechins can be allelochemical candidates in tea. However, the contribution of each compound to the total inhibitory activity of tea and their action pathways has not been clarified so far. Our study was conducted to evaluate the allelopathic potential of several tea samples from Vietnam.

The objectives of this study were: (i) to screen of allelopathic activity of tea leaves, (ii) to identify caffeine and evaluate the contribution of caffeine in the allelopathic activity of tea leaves, (iii) to evaluate the plant growth inhibitory activity of caffeine on the growth of crop and weed species, (iv) to evaluate the phytotocxic activity and concentration of caffeine from tea residues in three kinds of the soils and tea garden soil, (v) to screening of allelopathic potential of callus of Yabukita tea, Tsubaki and Sazanka and identify caffeine from callus of them.

All tea samples have shown a dose-dependent inhibitory effect on the radicle and hypocotyl growth of lettuce seedling. Among the seven tea samples, the leachates from Vina tea-green tea showed the highest inhibition on the radicle growth of lettuce seedlings with 50% suppression at 0.12 mg dry leaves/ml of agar, it is suggested that the highest inhibitory activity on the growth of lettuce seeds was observed in Vinatea- green tea sample. In contrast, black tea had the lowest plant growth inhibitory activity (EC_{50}>10 mg/ml of agar) compared to green, oolong and dried tea leaves (unprocessed tea samples).

The highest content of caffeine was found in the green tea samples (20.7-38.2 mg/ml) with the maximum content in Vinatea-green tea. The lowest caffeine content (20.7 mg/ml) was found in dried tea leaves. The difference in caffeine concentrations of tea samples may be related to the time of tea harvesting, variety, and the manner of tea processing. Caffeine had the specific activity EC_{50} of 75 and 183 µg/ml for lettuce radicle and hypocotyl growth respectively. Based on the value of the total activity, the contribution of caffeine can explain inhibitory activity by crude tea extracts in group of green and oolong tea sample. And for dried leave and Vinatea- black tea, we assume the possible effect of also other compounds such as catechins and polyphenols.

Moreover, the allelopathic activity of pure caffeine and aqueous tea extracts was highly selective on the growth of different plant species. Caffeine has been shown to have a strong allelopathic potential on seed germination and growth of some weeds and crops.

The recovery concentration of caffeine extracted with methanol from soil treated pure caffeine and green tea residue with EC_{50} value were about 254 ppm and 169 ppm in Fluvisol soil, and 746 and 663 ppm in Andosol soil, respectively. These results indicated that caffeine was absorbed by Fluvisol soil in a significant amount compared to Andosol and Sand soils. This observation perhaps due to the physical properties of soil and particularly its texture. Among three soils, caffeine concentration from tea residue was the highest in Andosol. Therefore, soil type significantly affects allelopathic activity.

The caffeine concentration in soil has a positive correlation with radicle growth inhibition of lettuce seeds at ($r=0.716$***; $p < 0.01$). The concentration of caffeine found from tea farm soil was 0.14 µg/g soil. Detection of caffeine in soil indicated that caffeine could directly affect the growth of plants in soil and the effect depends on concentration.

In addition, the study of allelopathic activity at the cellular level using the main Japanese tea cultivars. Callus of Yabukita tea showed strong inhibitory effect with the rate of 85% at 10 mg of both dried leaves and callus per 10 ml of agar on the growth of lettuce seedling. It was found that caffeine concentration in Yabukita callus was 19.2 µg/g.

The present study revealed that caffeine is responsible for the allelopathic effect of tea extracts and the selective weed inhibitory properties of aqueous extraction of Vinatea-green tea and caffeine. Addition, soil type significantly affects caffeine allelochemical activity. Although, caffeine concentration was found in a small amount, but our study has proved the presence of caffeine in the soil of the tea garden and in callus suggesting it could be agriculturally significant for sustainable weed management.

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ANOVA	Analysis of Variance
EC_{50}	Maximal Effective Concentration
HPLC	High Performance Liquid Chromatography
EGCG	(-)- Epigallocatechin gallate
ECG	(-)- Epicatechingallate

CHAPTER I. INTRODUCTION

1. Background

Chemical herbicides still play an important role in agricultural production, helping to improve labor for farmers, reducing weed pressure, are used in large quantities and popular due to their low cost and crop yields increased (Gianessi and Reigner, 2007). However, the overuse of herbicides has led to many harmful effects on production, the environment, public health, threatening the sustainable development of agriculture (Bukowska *et al*., 2006; Ayoola *et al*., 2008). Moreover, most herbicides could not suppress all weeds and reliance on herbicides has led to the emergence of resistance among many weed species (Service, 2013). Therefore, in recent years, allelopathic species and allelochemicals have been utilized as alternative weed management strategies in sustainable agricultural practices. Also, there are increasing possibilities for developing bio-herbicides from plants that could be used for weed control to minimize the heavy reliance on synthetic herbicides (Uddin *et al*., 2010; Mitchell *et al*., 2001; Dayan *et al*., 2015; Singh *et al*., 2003).

These public concerns over the impact of synthetic herbicide are forward to alternative weed control technologies based on natural products. In the search for natural chemicals useful as herbicides, a realistic assessment of whether natural phytotoxins might play a role in allelopathy, based on this biological activity (total activity), cyanamide was isolated from *Vicia villosa* (Kamo *et al*., 2003). Discovery of coumarin as the predominant allelochemical in *Gliricidia sepium* (Takemura *et al*., 2013). L-3,4-dihydroxyphenylalanine (L-DOPA) was isolated from Velvet bean (Fujii *et al*., 1991), and rutin from buckwheat (Golisz *et al*., 2007).

Allelochemicals are released into the environment through root exudation, volatilization, leaching or decomposition of plant residues in soil and may inhibit the germination and the growth of competing plants including weed (Weir *et al*., 2004; Xuan *et al*., 2005). The reason for the current trend in the exploitation of allelopathic plants and allelochemicals could be due to the potential of finding new environmentally friendly bioactive compounds (Reigosa *et al*., 2006). Natural herbicides from allelopathic compounds will have some important advantages over the commercial herbicides because they could be biodegradable (Macías, 1995; Duke *et al*., 2000). The study of the phytotoxic potential offers useful clues in the investigation of new models of natural herbicides that could be more specific and less harmful than the synthetic substances used in agriculture. Allelochemicals from sunflower leaves caused an increase in lipid peroxidation, loss of seed germinability may be associated with membrane lipid peroxidation, detected as increased malondialdehyde content (Bogatek *et al*., 2006). The phytotoxicity of L-dopa is considered due to the oxidative damage caused by reactive oxygen species and/or free radical species (Hachinohe and Matsumoto, 2007).

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid which is known for its medicinal properties and also recognized for its essential roles in allelopathic effects against plant species (Rizvi *et al*., 1981), and chemical defense against pathogens and herbivores (Kim *et al*., 2008). Besides, caffeine is a relatively safe compound for humans classified by the US Food and Drug Administration (US Code of Federal Regulations 2001). The highest amount of caffeine has been found in guarana (4-7% of dry weight) followed by tea leaves (3.5%) and coffee bean (1.2-2.2%) (Clifford *et al*. 2000). According to Takeda *et al*. (1994) the concentration of caffeine determined in tea was the highest compared with the other plants such as coffee, cacao, mate, and among others. Caffeine inhibits the growth of rice and maize seedlings and the effect is more severe on roots than on shoots (Smyth 1992; Anaya *et al*., 2002). Caffeine from seeds of *Coffea arabica* has been demonstrated to have selective phyto-toxicity against weeds such as *Amaranthus spinosus* (Rizvi *et al*., 1981).

Tea (*Camellia sinensis* (L.) Kuntze) produces a wide range of secondary metabolites of diverse chemicals structures, such as alkaloids, flavonoids, phenols, steroids and terpenoids (Hadacek, 2002). Among these categories of secondary metabolites, alkaloids are a large group of nitrogen-containing organic compounds, which are toxic to insects, herbivores, fungi, viruses and can inhibit the growth of bacteria and plant seedlings. Alkaloids are also considered defense chemicals, and many plants produce them to avoid the attacks of insects (Wink, 1998). Liu and Lovett (1993) suggested that the presence of the two alkaloids, gramine and hordenine of barley (*Hordeum vulgare* L.) may play a significant role in self-defense by the crop.

Tea has been known to have allelopathic potential. Dibah *et al*., (2012) demonstrated the high allelopathic effect of water extracts of very young tea leaves at concentrations of 3 and 5% on seed germination and seedlings growth of *Vicia sp*. According to (Rezaeinodehi *et al*., 2006), the germination and growth of garden cress (*Lepidium sativum*), lettuce (*Lactuca sativa*), redroot pigweed (*Amaranthus retroflexus*) and golden foxtail (*Setaria glauca*) were stimulated at low concentrations and inhibited at high concentrations of tea extracts (leaf, flower and fruit). The authors assumed that the phytotoxicity effect can be due to the presence of soluble allelochemicals in the extracts. Besides, leaf area and dry weight of redroot pigweed and golden foxtail after 40 days planting were strongly decreased under the effect of the dried residue of tea at rates of 24 and 32 g kg^{-1}. Moreover, an aqueous extract of tea residues significantly affected the germination of wheat and maize at 100mg/ml. Thus, tea extract can be applied to inhibit the growth of weeds in agriculture (Waris *et al*., 2016).

2. Allelopathy and allelochemicals

Allelopathy

The term "allelopathy'' was defined by Rice (1984) as "any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment". In 1996, the International Allelopathy Society broadened its definition of allelopathy to refer to any process involving secondary metabolites produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals) including positive and negative effects (IAS, 1996).

Finding alternatives to synthetic chemicals in the weed control which tend to develop herbicides have plant origin is a necessary and feasible direction. The phenomenon of allelopathy has been suggested to be one of the promising alternatives and has the potential for weed control. The advantage of allelopathy has proved that the reducing of dependence on synthetic herbicide and achieving much more sustainable agriculture.

Various studies have been done to explore the inhibitory potential of different allelopathic crops and trees for weed management (Cheema *et al*., 2004; Iqbal *et al*., 2007; Jamil *et al*., 2009, Farooq *et al*., 2011b).

Allelochemicals

Allelopathic plant releases chemicals into the surrounding environment termed as allelochemicals. The allelopathic nature of any plant is due to the presence of different types of allelochemicals in it. Allelochemicals can be present in any part of the plant they can be found in different concentrations in each organ (Gatti *et al*., 2010). In the process of competition and development, the plant produced more organic compounds called secondary metabolites, also known as natural products or allelochemicals which may inhibit germination, growth and development of other plants including weeds. These allelochemicals from plant are released into the environment via the exudation from the roots leaching from leaves and other aerial plant parts, volatile emissions, and the decomposition of plant material (Einhellig and Leather 1988; Weir *et al*., 2004; Xuan *et al*., 2005). Important secondary metabolites identified as allelochemicals are phenolics, alkaloids, flavonoids, terpenoids, momilactone, hydroxamic acids, brassinosteroids, jasmonates, salicylates, glucosinolates, carbohydrates and amino acids (Kruse et al., 2000; Jabran and Farooq, 2012).

Allelochemicals leached from the aerial parts of donor plants finally enter the soil (Fig 1.1). The chemicals may reach other plants (receivers) through transport from the donor plants in the soil and may induce the inhibitory or stimulatory activity on the receiver plants. The behavior of allelochemicals in soil is affected by the soil physicochemical properties and soil organic matter and organisms. They impact the main processes of adsorption, desorption and transport in soil and the metabolism of allelochemicals.

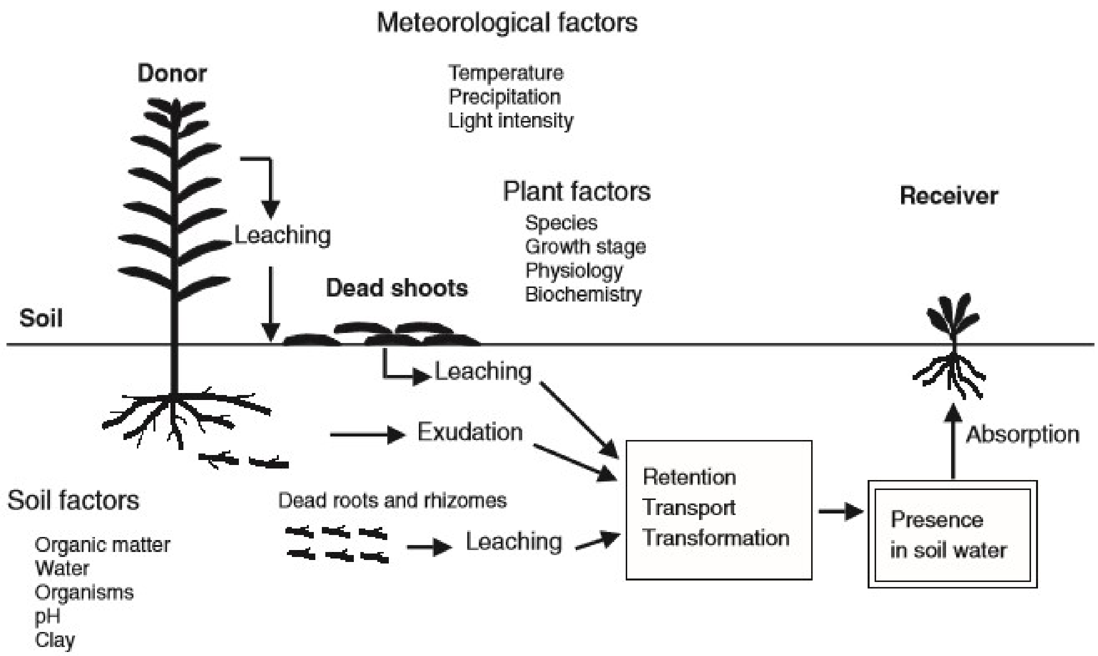


Figure 1.1. Factors affecting behavior and the phytotoxic activity of allelochemicals in soil (Kobayashi, 2004)

Evaluation of the phytotoxic activity of allelochemicals under natural conditions and understanding how allelochemicals can effect on plant growth in soil is necessary for further study in the real condition. In the presence of the soil, the inhibitory activity of allelochemicals on the growth of plant became weaker, since adsorption and transformation reactions as well as allelochemical decomposition by microorganisms caused the disappearance of allelochemicals in soil. All reactions prevent the deposition of allelochemicals in soil. The plant-growth-inhibitory activity of L-DOPA was reduced by the application of soil (Furubayashi *et al*., 2005). Yamamoto (2009) demonstrated that the recovery rate of coumarin in Cambisols was higher than Andosols soil, suggesting that the phytotoxic activity of the allelochemicals was affected by soil properties. The concentration of L-DOPA in the soil solution decreased quickly by adsorption and transformation reactions. The rate of L-DOPA transformation was faster at higher pH values and the transformed products from L-DOPA consisted of specific components with low molecular weight when L-DOPA was transformed at a pH value of 9.7 or higher (Hiradate *et al*., 2005). The dynamics of juglone content in black walnut-maize (*Zea mays*) alley cropping system varied at phytotoxic levels and also depended upon the microbial ecology of soils (Jose and Gillespie, 1998).

Allelochemicals affect the physiology of crop plants. It can inhibit plant growth, alter mineral uptake, cause stomatal closure and induce water stress, influence respiration, affect photosynthesis and protein synthesis impair hormone balance and alter enzyme activities. Cruz *et al*. (1998) investigated the effect of allelochemicals on the *P. vulgaris* root where root tip cells were extruded together, and that cell organization was disordered with little cell differentiation. In another study, Baziramakenga *et al*. (1997) studied the allelochemical effect on *Glycine max*. He found that soybean plant growth is affected by the interference of allelochemicals with nucleic acid and protein metabolism.

Allelopathy could be applied directly for weed control particularly crop plants (crop plants with allelopathic potential can be used as cover, smother, and green manure crops) or can be used indirectly as natural herbicides (changing of structural or synthesizing of chemical analogs with improved activity (Vyvyan, 2002; Singh *et al*., 2001). Application of allelopathic water extracts such as sorghum water extract controlled *Chenopodium album*, *Phalaris minor*, *Fumaria indica* and *Rumex dentatus* in wheat crop. In particular, it reduced from 15 to 47% in weed density and from 19 to 49% in dry weight (Cheema and Khaliq, 2000).

The combined application of "sorgaab" with sunflower, eucalyptus, sesame, brassica and rice water extracts is more effective for weed management than the sole application of either water extract (Cheema *et al*., 2003). Allelopathy can play an effective role in controlling weeds through soil incorporation of allelopathic crop residues, for example, mulching of residues also suppresses weed flora (Cheema *et al*., 2003; Khaliq *et al*., 2010).

Mode of actions of allelochemicals include various effects such as (a) on division, elongation and ultra-structure of cells, (b) hormone-induced growth, (c) on membrane permeability, (d) on mineral uptake, (e) effects on photosynthesis, (f) effects on respiration,

(g) effects on protein synthesis, lipid and organic acid metabolism, (h) inhibition or stimulation of specific enzyme activity, (i) effects on water relationship, (j) effects on DNA or RNA synthesis (Rice, 1984).

Specific and total inhibitory activity

There are numerous allelochemicals candidates which have been reported; however, not all of them were to clarify the most dominant allelochemical in plant as well as evaluation of the contribution of those allelochemicals act truly to allelopathic potential. Total activity method applied for this calculate purpose, total activity also revealed the strong or weak allelopathic activity of each plant.

The biological activity of a compound expressed by EC_{50} is the effective concentration of a compound to induce half of the maximum action. This activity is expressed by the specific concentration of the compound and is termed as "specific activity" (Hiradate, 2004; Fujii & Hiradate, 2005). The total activity is a function of the specific activity and total content of the examined compound in the organism. It is the concentration of the compound in the plant per specific activity (EC_{50}). As the concentrations of the compound and the specific activity have the same dimension, the total activity is a number without any unit:

Total activity = (concentration or content) / specific activity (EC_{50})

A compound with high total activity does not necessarily have a high specific activity (low EC_{50}). The total activity is a useful indicator to reveal the allelopathic potential of a compound (Hiradate, 2006).

A preliminary experiment on tea (*Camellia sinensis*) residue extract has previously shown allelopathic potential on some weeds and crop species (Rezaeinodehi *et al*. 2006; Dibah *et al*. 2012; Waris *et al*., 2016). However, the contribution of bioactive compounds in tea and their total allelopathic activity, as well as the evaluation of caffeine as a putative allelochemical, have not yet been studied. Moreover, identification and isolation of active compounds in allelopathic plants are necessary to meet the needs for development new herbicide. Although many studied indicated caffeine as an allelochemical candidate, relatively little is known about the evaluation of caffeine allelochemical in tea the by total activity approach and their action pathways have not been clarified so far.

3. Objectives

The objectives of this study were:

(i) To screen of allelopathic activity of tea leaves.

(ii) To identify of caffeine and evaluate the contribution of caffeine in the allelopathic activity of tea leaves.

(iii) To evaluate plant growth inhibitory activity of caffeine on the growth of crop and weed species.

(iv) To evaluate the phytotoxic activity and concentration of caffeine from tea residues in three kinds of the soils and tea garden soil.

(v) To screen of allelopathic activity of callus from tea.

CHAPTER II. LITERATURE REVIEW

2.1. Biological characteristic of tea plant (*Camellia sinensis* (L.) Kuntze)

Kingdom: Plantea

Division: Magnoliopsida

Ordo: Ericales

Familia: Theaceae

Genus: Camellia

Species: *Camellia sinensis*

(Tat Loi. 2001)

Figure 2.1. *Camellia sinensis* (L.) Kuntze**. (**http://tracuuduoclieu.vn/che.html)

The tea plant is a shrub and native to Southeast Asia but is currently cultivated in more than 30 countries around the world, mainly cultivated in China, Vietnam, India, Japan, Taiwan, Sri Lanka, Indonesia and in central African countries. *C. sinensis* is the species of plant whose leaves and leaf buds are used to produce tea, small tree that is usually trimmed to below 2 m. It has a strong taproot. The leaves are 4–15 cm long and 2–5 cm in broad. The flowers are yellow-white, 2.5–4 cm in diameter. Tea plants prefer a cool and humid climate and the mountain conditions are optimum to tea plant growth (Ho *et al*., 2008).

Vietnam is not only famous as the home of rice but also known as one of the most ancient cradles of tea. Vietnam has many natural advantages to cultivate and process tea, such as tropical climate (temperature from 18-23 ºC and humid >80%), Feralitic soil with a pH of 5.5-7. Tea plantations in Vietnam are concentrated mainly in the north. Besides being one of the solutions to eliminating hunger and reducing poverty, tea plantations also help protect the environment and bring high income to the growers. Consequently, tea has become a high-value product of Vietnam.

In Vietnam, tea is mostly grown in the northern highlands at latitudes Thai Nguyen and Lam Dong provinces (Fig 2.2) are representative of these two major tea producing regions in Vietnam.

In recent years, the area of tea cultivation and the number of manufacturers has increased rapidly. Particularly, from 5,400 hectares in 1975, in recent years, the area of cultivation has reached to 130,000 hectares. The average yield is 8 tons of fresh buds/ha and at No. 5 position in the world in the export of tea in 2018 (Vietnam exporting and importing report, 2019). However, the price of Vietnamese tea products is lower in comparison to that of other countries due to limited processing technology. In addition, there is no utilization of the remaining tea leaves which are of low standards for the processing of high-quality tea products for domestic consumption or export; remaining tea leaves have become wastes, leading the effective of tea cultivation is low. From this fact, there is a possibility of using this tea raw material as a fertilizer or a herbicide for weed control that is available not depending on harvest season.

Figure 2.2. The geographical location of the two major tea growing regions in Vietnam (Vuong *et al*., 2011)

2.2. Chemical composition of tea

Major chemical constituents of tea are presented with their composition in Table 2.1. (Mohanpuria *et al*., 2010).

In tea, polyphenols, catechins and caffeine are the most active and dominant constituents, catechins and caffeine are secondary metabolites that serve as defense compounds in plants. They provide plants with resistance to pathogens and predators (Ames *et al*. 1990), moreover tea leaves are an important source of amino acid, minerals, protein, lipid and other elements which are essential to human health.

Table 2.1. Major chemical constituents and their composition in fresh tea leaves (Mohanpuria *et al*., 2010)

Constituent	Content (% dry weight)
Polyphenols	30 - 40
Caffeine	3 - 6
Amino acids	4 - 5
Proteins	14 -17
Polysaccharides	14 - 25
Lipids	2
Organic acids	1 - 5
Minerals	5 - 6

2.2.1. Polyphenols

Polyphenols in tea are well-studied bioactive compounds; they are constituents that have a decisive role in the quality and pharmacological of tea. Polyphenols are widely distributed in plants, but are found in relatively high amounts in tea, the dominating polyphenolic compounds found in tea are flavonols or flavones (Wang *et al*., 2005).

Important features of the antioxidant are to reducing free radicals, these free radicals can oxidise nucleic acid, protein, lipid, DNA, and cause degeneration diseases, oxidation resistant compounds as phenolic acids, polyphenols, flavonoids, will acquire the free radicals (peroxide, hydroperoxide or peroxyl lipid) which extinguished this oxidation process itself (Molyneux, 2004; Sharma, 2009). Polyphenols exhibit antimicrobial activity. Some studies have investigated the effects of polyphenols on intestinal pathogens. Polyphenols can inhibit the growth of clostridia and Helicobacter pylori but not of some intestinal lactic bacteria (Gramza *et al*., 2005). Besides, antioxidants property of polyphenols is applied for preservation in food which has great potential even can make use of older tea leaves.

2.2.2. Catechins

The beneficial effects of tea have been mainly attributed to catechins that have an important role in the creation of color, odor and taste the tea products. The antioxidant properties of catechins depend on the number and position a hydroxyl group in the molecules and consequently binding and neutralization of free radicals by these hydroxyl groups (Farkas *et al*., 2004 and Guo, 1999).

The major catechins present in tea leaves are (+)-catechin (C), (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), and (-)–epicatechin (EC) (Fig 2.3) (Femandez *et al*., 2000). EGCG with eight – OH groups determining its high antioxidant activity is the major catechin in tea accounting for more than 10% on a dry weight basis, while other catechins are present in smaller amounts; catechins are the most abundant group of phenolic compounds in fresh leaf and green tea (Hashimoto *et al*., 1987). The water solubility of EGCG is about 521.7 g/ml while that of caffeine is 21.7 g/ml. Besides, catechin is a potent phytotoxin that causes plants to self-destruct by producing free radicals and by triggering genes that kill the cells (Bais et al. 2003).

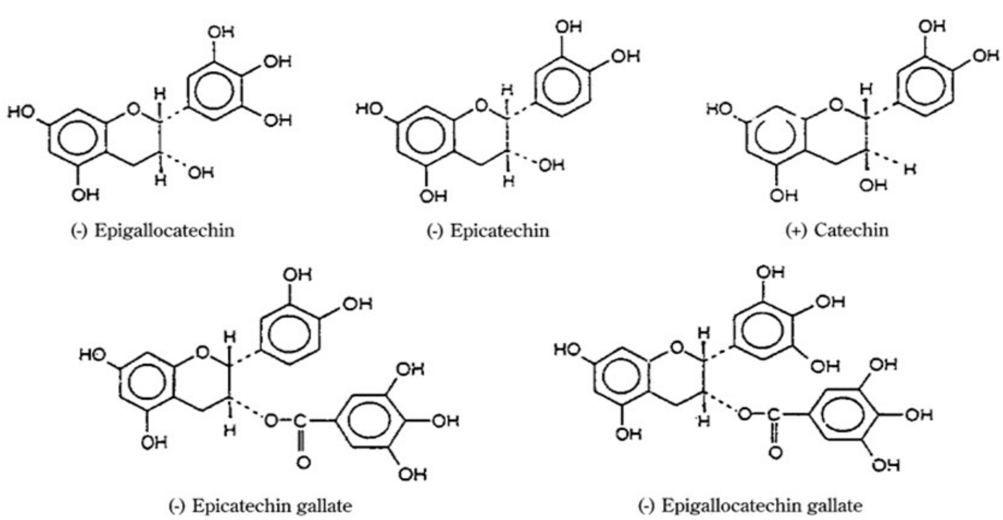


Figure 2.3. Structure of catechins (Femandez *et al*., 2000)

2.2.3. Caffeine

Caffeine (1,-3,-7-trimethyl xanthine, C8H10N402), is a class of naturally occurring compounds containing nitrogen and having the properties of an organic amine base (alkaline, alkaloids) (Fig 2.4) (Mohammed *et al*., 2009). Along with other methylxanthines, including theobromine (3,7-dimethylxanthine), paraxanthine(1,7-dimethylxanthine) and methyluric acids, caffeine is a member of a group of compounds known collectively as purine alkaloids. In its pure form, caffeine is a white crystalline powder and a stimulant drug that tastes very bitter; this is the reason for preventing insects and herbivores from feeding plants (Rebecca *et al*., 2004).

Among the purine-alkaloid containing plants, most studies have been carried out with species belonging to the genera *Camellia* and *Coffea*. Caffeine is a central nervous system and metabolic stimulant and is used both recreationally and medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs (Nehlig *et al*., 1992).

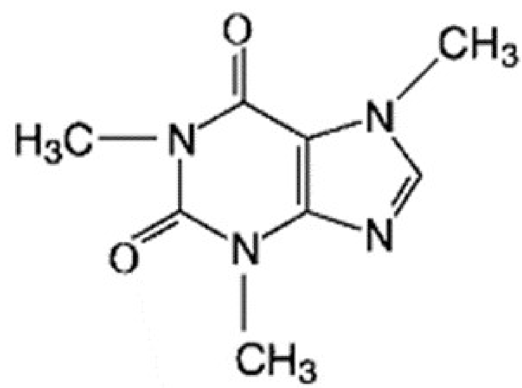


Figure 2.4. Structure of caffeine (Mohammed *et al*., 2009**)**

Caffeine is a typical purine alkaloid, along with other compounds present in tea leaves; caffeine is believed to involved two major roles: in allelopathy and plant defense. Rizvi and Rizvi (1992) suggested that caffeine might be a good candidate for developing new natural product-based herbicides because caffeine exerted a differential action on several plant species

Caffeine negatively affects the rooting potential of hypocotyl cuttings of Phaseolus. aureus, the rooting potential declined with increasing concentration (Batish, *et al*., 2008). Chou *et al*. (1980) pointed that, caffeine was predominant among phytotoxins compounds identified in *Coffea arabica*. It was distributed in fallen leaves, roots, and young seedlings and young coffee seedlings may produce enough phytotoxic amount and release to the soil to suppress the growth of weeds competing with them. Friedman *et al.* (1983) tested effects of exogenous caffeine on mitosis in root tips of seedlings of coffee and cell division was inhibited in root tips that were exposed to 10 mM caffeine for 24 hours. Other study by Rizvi *et al*., (1987) demonstrated the significant decrease in amylase activity during germination of seeds of *Amaranthus spinosus* when they were treated with 1,000 ppm caffeine.

According to Mostakim *et al*. (2014), it could be indicated that caffeine proving to be a successful botanical pesticide for the management of the red flour beetle and other storage pests. Hollingsworth *et al*. (2002) has found that caffeine solution can kill snails and slugs; large slugs were killed by spray applications containing 1–2% caffeine. Using caffeine is effective because it is very safe natural product and it is proved by the US Food and Drug Administration. In an agricultural environment, slugs and snails can be more sensitive to leaching poisoning from caffeine than other animals, demonstrating that caffeine has pesticide activity with species selectively.

2.3. Tea productions

Nowadays, many types of tea have been produced but generally, teas are classified into three major types: no fermented green tea, semi - fermented oolong tea; and fully fermented black teas (Lin *et al*., 1998). These basic types of tea have different quality characteristics, including appearance, flavor, taste, and color. The composition of polyphenols catechins, caffeine, in commercial teas varied depending on species, season, and horticultural conditions and, particularly, with a degree of fermentation during the manufacturing process (Zuo *et al*., 2002). And for Vietnamese tea products, they are manufactured and controlled under Vietnam quality standards that mean no addition of artificial caffeine or catechins to tea products.

2.3.1. Green tea

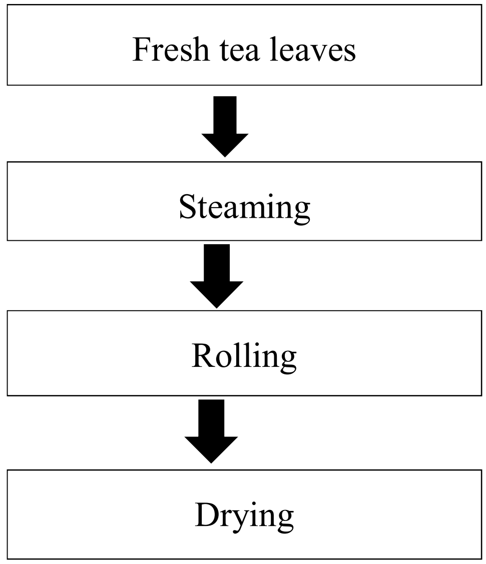


Figure 2.5. Processing procedure of green tea (Hilal and Engelhardt, 2007)

After being plucked, fresh leaves of tea immediately treated with high temperatures in a factory in order to inactivate oxidizing enzymes of leaves. For high green tea, free amino acids, caffeine and polyphenols are important components. Especially, catechins, the main component of polyphenols, are well known for their antioxidant properties and were evaluated in the prevention of many diseases related to free radicals, such as cancer, cardiovascular and neurodegenerative diseases (Mukhtar *et al*., 2000; Mandel *et al*., 2004). The levels of EGCG and total catechins were determined in the order green tea > oolong tea > fresh leaves > black tea (Lin *et al*., 1998) and caffeine content in green tea infusions was between 141and 338 mg/l (Reto *et al*., 2007).

2.3.2. Oolong tea

The fermentation process, oxidizing enzymes are especially active and change tannin and other substances in raw leaves to the oxidized forms. As a result, the color of the leaves become typically brown or red and their taste and aroma enhanced as in black tea and oolong tea. In oolong tea, EGCG and ECG are considered the major contributors to the antioxidant activities and belong to moderate level of enzymatic oxidation. Yen and Chen reported that oolong tea exhibited a stronger antimutagenic activity than green or black tea (Yen and Chen, 1994). The results demonstrated that the level of EGCG and catechins in oolong tea is lower in green tea, but higher than black tea. Zuo *et al*. (2002) also have observed much reduced caffeine level in oolong teas especially Fujian oolong tea.

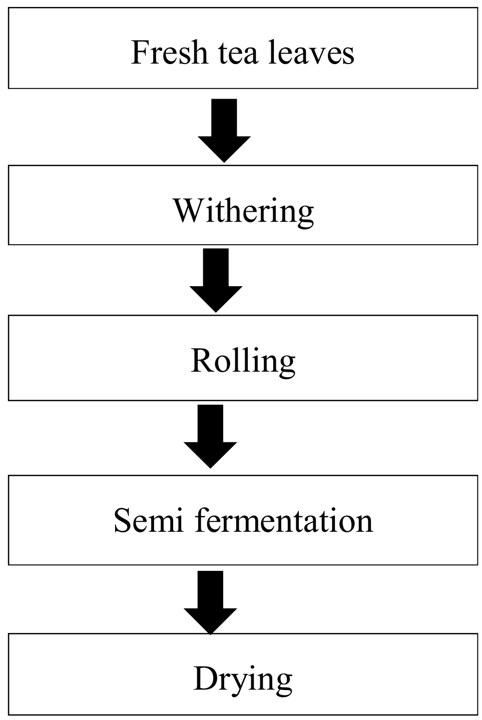


Figure 2.6. Processing procedure of oolong tea (Hilal and Engelhardt, 2007)

2.3.3. Black tea

The fermentation has little effect on caffeine in the leaf (Lin et al., 1998) and it is suggested, that caffeine content in tea leaves depends on two factors during the fermentation: the growth of microorganisms and the tea composition. According to Wang *et al*. (2005) and Wang *et al*. (2008), the fermentation increases in caffeine content and decrease the content of antioxidants. In black tea, almost catechins forming are oxidized and polymerized to theaflvins and thearubigins which are responsible for the color, flavor and brightness of tea, so black tea is a valuable source of polyphenols (McDowell *et al*., 1990).

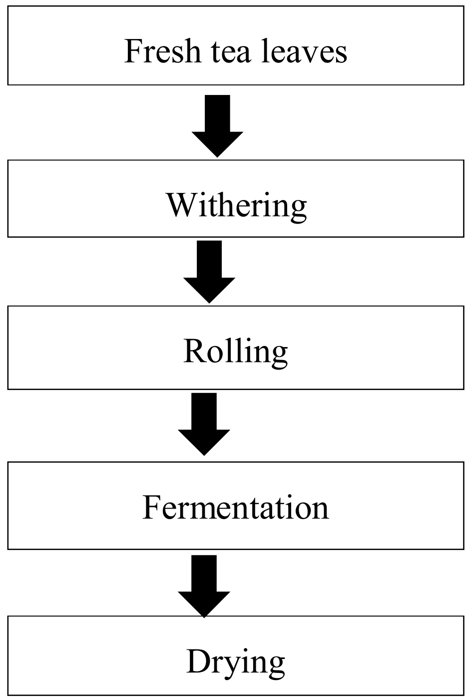


Figure 2.7. Processing procedure of black tea (Hilal and Engelhardt, 2007)

The comparison of Vietnamese tea with several tea types of tea from other countries the results indicated that, Vietnamese green tea had a lower content of caffeine compared to Chinese green tea but higher caffeine level than Japanese green tea. Vietnamese oolong teas had a lower amount of catechins, but a similar level of caffeine compared to Chinese oolong teas. Vietnamese black tea samples had a lower content of catechins and caffeine than the other black teas available in Australia (Vuong, *et al*., 2011).

CHAPTER III. SCREENING OF ALLELOPATHIC ACTIVITY FROM TEA

3.1. Introduction

To determine if a plant species might influence its neighboring plants by allelopathy, many preliminary studies that can indicate allelopathy by growing the receiver plant with plant tissue residues (living material, dried biomass, or leaf litter) of the donor plant (Batish *et al*., 2009). Or simply growing the receiving plant in pots in the presence of different numbers of donor plants. However, the previous preliminary studies need extraction procedure, and suitable for a small number of samples.

As for the leaching from plant material, the inhibitory effect is widely tested by the 'Sandwich method', which replicates the environmental conditions at the laboratory level (Fujii *et al*., 2003) this method lets the allelochemicals to move to agar medium, and can screen a large number of samples from leaf litter leachates (Fujii *et al*., 2004; Appiah *et al*., 2015; Mardani *et al*., 2016).

Therefore, the present experiment was done to evaluate the allelopathic activity of leaching from tea leaf.

3.2. Materials and Methods

Materials

Fresh tea sample (V1) was collected from Thai Nguyen province, Vietnam in 2014 December, and dried at 60^{o}C for 14 hours.

Six types of tea obtained from different commercial brands in Vietnam were used: Vinatea -green tea) (V2), Vinatea -oolong tea (V3), Thai Binh – Lang Son (oolong tea) (V4), Duc Thien (green tea) (V5), Kim Anh (green tea) (V6) and Vinatea- black tea (V7). (Fig 3.1)

V1. Dried tea leaves

V2. Vina- green tea

V3. Vina - oolong tea

V4. Thai Binh – Lang Son oolong tea

V5. Duc Thien - green tea

V6. Kim Anh filter bag tea

V7. Vina- black tea

Figure 3.1. Picture of seven tea samples

Tea products were classified into three major types: non-fermented (V2, V5, and V6); semi-fermented (V3, V4) and fully fermented (V7). The classification was based on the different processing of tea types at manufacture (Table 3.1). V2, V5 and V6 belong to group of green tea, V3 and V4 belong group of oolong tea, and V7 is black tea

Table 3.1. The classification of tea types (Tat Loi. 2001)

Tea sample	Variety	Place of collection	Time of harvest	Drying
V1	Trung Du	Thai Nguyen	September	60°C for 14 hours
V2 (Green tea)	Trung Du	Thai Nguyen	From July to August	100-110°C for 8 minutes for the first time, the second time at 80-90°C for 7 minutes.
V3 (Oolong)	Kim Tuyen	Moc Chau-Son La	From July to August	90-95°C for 24 minutes
V4 (Oolong)	Oolong-ThanhTam	Lang Son	From July to August	90-95°C for 24 minutes
V5 (Green tea)	San Tuyet	Yen Bai	From July to August	100-110°C for 24 minutes.
V6 (Green tea)	Trung Du	Ha Giang	From July to August	90-100°C for 24 minutes
V7 (Black tea)	PH1	Phu Tho	From May to October	105-110°C for 25-30 minutes

Screening of phytotoxic potential of tea samples by the Sandwich method

The phytotoxic potential of seven tea samples (V1-V7) from Vietnamese tea brands was tested using the sandwich method as described by Fujii *et al*. (2003). Samples of oven-dried tea leaves (0.5, 1, 2, 5, 10, and 50 mg) of each dried tea leaf samples were placed in each well of the six-well multi-dish plastic plates. Commercially available agar (Nacalai Tesque Co. Ltd., Japan gelling temperature 30–31 °C) was prepared at the concentration of 0.75% (w/v) and autoclaved at 115 °C for 15 min. The autoclaved agar was cooled down to 45 °C in a water bath, and 5 ml of agar was added to each well of the multi-dish plastic plate. After gelatinizing the agar within 30–60 min at room temperature (25 °C), another 5 ml agar was added to each well of the multi-dish plate; this made two layers of agar on tea samples. Five lettuce seeds were then placed on the surface of the agar. The multi-dish plates were covered with plastic tape and kept in the incubator for three days at 22 °C in complete darkness. After three days, radicle and hypocotyl lengths and germination percentage of seeds were recorded (Fig 3.2). Treatments with agar solution were used as the control, and each experiment was conducted three times and presented as the mean of three replicates. The EC_{50} values were determined by a logistic regression equation of the concentration–response curves.

Statistical analysis

The data were statistically analyzed using XLSTAT software for Mac version 2019.1.2 (Addinsoft, Paris, France). Analysis with a confidence interval of 95% was conducted with one-way analysis of variance (ANOVA) followed by Tukey & Dunnett multiple comparisons.

3.3. Results

Screening of allelopathic potential of tea samples using a Sandwich method.

The inhibitory effects of leachates from tea leaf samples on the radicle and hypocotyl growth of lettuce seedlings is shown in Table 3.2. Radicle elongations of lettuce seedling were inhibited more than hypocotyl elongations at a concentration from 0.1 mg/ml to 5 mg/ml of tea samples in a group of green tea and oolong teas. It was also found that group of green tea and oolong tea showed the strongest inhibitory activity on lettuce seedling by radicle elongation in the range of 3.45-5.27% for 5 mg/ml treatment.

Among the seven tea samples, V2 had the lowest EC_{50} value (the strongest inhibitory activity) on the radicle and hypocotyl growth of lettuce seedlings at 0.12 and 1.00 mg/ml, respectively. The highest EC_{50} (weakest inhibitory activity) of 1.90 and 5.00 mg/ml for the radicle and hypocotyl growth of lettuce seedling, respectively, was shown by tea sample V7. The stimulatory effect of leaves of V2 and V7 samples leaching on the hypocotyl growth of lettuce seedling was observed at lower concentration (0.05-0.5 mg/ml).

3.4. Discussion

The growth of lettuce seedlings was more highly suppressed by the tea samples from V2 to V6 than tea samples V1 and V7 based on the EC_{50} values. Also, the hypocotyl growth of lettuce seedlings was less affected by the leachates from tea leaves than radicle. Allelopathy could occur through the production and release of many different chemical groups with effects (inhibitory or stimulatory) on other organisms in the environment. These chemical classes are mainly phenolics, alkaloids, and terpenoids, and some of these compounds are promising allelochemicals (Soltys *et al*., 2013). However, the phytotoxic effect of a plant species can be due to the combined effect of a mixture of compounds rather than a single constituent (Wink, 2013). Although the inhibitory effect of tea samples on the lettuce seed germination was not detected, the difference in inhibitory potential of all tea samples on the radicle and hypocotyl growth of lettuce seedlings can be explained by various compounds such as alkaloids, polyphenols (particularly flavonoids), and phenolic compounds (Macias *et al*., 1996). In parallel with Chatterjee *et al*. (2013), green tea was found to have markedly higher phytotoxic activity than black tea as green tea contains more flavonoids (catechins).

The presence of these soluble compounds may also be responsible for the observed phytotoxic activity of tea, and the inhibitory effect exhibited may be due to the presence of putative allelochemical. The relatively higher inhibition found in radicle than in hypocotyl of lettuce seedling due to the direct contact between the root and phytotoxic compounds present in agar medium (Salam and Noguchi, 2010; Morikawa *et al*. 2012). Related to previous reports using sandwich method, strong growth inhibitory activity of several species had an EC_{50} ranging from 0.28 to 6.90 dry weight equivalent ml (Takemura *et al*. 2013; Appiah *et al*., 2015; Mukaromah *et al*. 2016), Vietnamese tea samples were estimated to have promising allelopathic potential based on the EC_{50} values of these tea samples in present study.

3.5. Conclusion

The percentage of elongation of radicle and hypocotyl of lettuce seedlings affected by leachates from 7 tea samples based on a sandwich method. The results indicate that increasing leaf dry weight enhanced the inhibition of radicle growth in lettuce seedlings. Comparing EC_{50} values, V2 was considered to have the highest allelopathic potential among 7 samples.

3.6. Tables and Figures

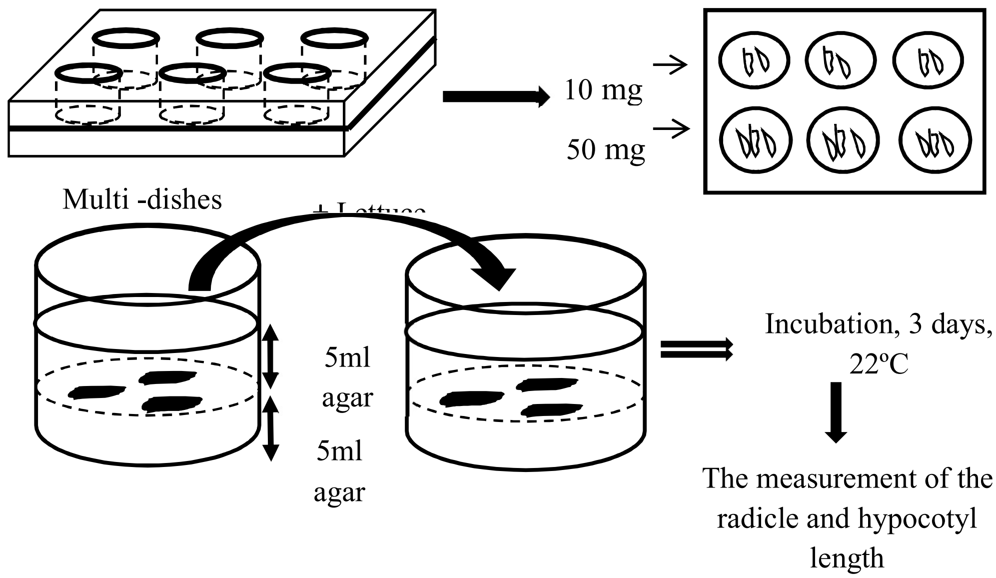


Figure 3.2. Sandwich method

Table 3.2. The effect of the phytotoxicity of tea samples on the growth of lettuce seedlings by the sandwich method

Samples	Type of tea		Concentration of tea samples (mg of dried leaves/ ml of agar) 0.05	0.1	0.2	0.5	1	5	EC_{50} (mg of died leave/ml of agar)
			Percentage of radicle growth of lettuce seedling compared to control						
V1	Fresh tea	Radicle	98.2±13.8	94.6±10.5	71.6±14.78	41.4±7.95	28.1±8.46	10.2±4.36	0.40[a]
		Hypocotyl	103±12.2	110±17.82	101±15.7	104±12.6	88.2±16.81	52.9±14.0	5.10[a]
V2	Green tea	Radicle	87.7±10.21	52.1±17.6	35.1±14.7	22.9±8.90	17.1±10.61	4.46±1.55	0.12[c]
		Hypocotyl	100±8.46	92.3±18.55	76.3±17.12	66.2±25.46	54.3±25.4	23.4±9.42	1.00[c]
V3	Oolong tea	Radicle	90.4±9.84	76.4±10.1	40.9±8.44	34.9±10.1	18.3±4.83	5.07±2.18	0.23[c]
		Hypocotyl	99.3±13.53	93.6±19.70	94.8±13.15	92.2±18.29	65.3±23.43	18.9±12.17	2.20[bc]
V4	Oolong tea	Radicle	85.5±11.41	53.1±16.2	35.9±9.22	31.5±7.69	20.5±5.63	5.27±1.37	0.17[c]
		Hypocotyl	98.5±14.1	101±14.45	95.7±21.78	87.4±18.75	67.9±15.82	13.7±7.47	1.40[bc]
V5	Green tea	Radicle	89.7±7.23	56.4±12.8	31.4±8.25	20.5±5.57	21.3±8.78	3.65±1.24	0.16[c]
		Hypocotyl	100±12.06	99.5±14.06	90.9±17.05	63.9±21.5	65.7±23.12	12.8±3.50	2.10[bc]
V6	Green tea	Radicle	88.7±8.24	68.5±11.13	35.9±18.1	22.9±5.38	17.1 ±3.38	3.45±1.06	0.21[c]
		Hypocotyl	104±10.3	92.7±15.62	84.3±21.44	84.7±12.80	51.6±13.63	11.9±2.81	2.20[b]
V7	Black tea	Radicle	90.6±13.3	80.5±14.18	72.3±12.58	57.5±13.17	59.7±16.14	13.4±3.74	1.90[b]
		Hypocotyl	106±15.7	112±20.7	100±17.41	113±16.24	107±20.1	50.1±14.6	5.00[a]

Values followed by the same letter within the same column are not significantly different ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replications ± standard deviation (SD) * EC_{50}: mg of dried leave/ml of agar. R; Radicle and H; Hypocotyl.

CHAPTER IV. IDENTIFICATION OF CAFFEINE LEACHING FROM TEA

4.1. Introduction

Caffeine is a naturally occurring chemical stimulant found in the leaves, seeds and fruits of a numerous plant species of a group of compounds called trimethylxanthine. Green tea is estimated to have more amount of caffeine among the other three tea leaves followed by white tea, black tea and red tea (Rebecca *et al*., 2014).

Caffeine can be isolated from teas with liquid-liquid extraction and quantitated by gas chromatography with nitrogen-phosphorus detection. A special ultrasound method with high-frequency, well penetrating power and the sound wave can be used to extract the caffeine. The quantity of caffeine is increased with temperature raised. (Sheu *et al*., 2009). Caffeine can be extracted more at the boiling temperature than at 30°C (Atomssa and Gholap, 2011).

The total activity approach is a function of both the specific activity and the total content of the bioactive compound(s) in plants and is defined by the concentration or content of a compound in a plant per specific activity. Specific activity is expressed by EC_{50}, which is the effective concentration of an examined compound required for 50% inhibition of a receptor plant. The extract of plants with high total activity has high potential to be influential as an allelopathic plant. It is a fundamental concept to evaluate the contribution of natural bioactive chemicals related to allelochemicals (Fujii and Hiradate 2005) and can be used as criteria to evaluate the most influential compound. Through the total activity approach, several compounds such as L-3,4-dihydroxyphenylalanine (L-DOPA), cyanamide, and rutin were successfully isolated and evaluated as the predominant compounds for the allelopathic activity of Velvet bean, hairy vetch, and buckwheat plants, respectively (Fujii *et al*., 1991; Kamo *et al*., 2003; Golisz *et al*., 2007).

In the previous chapter, the observed tea allelopathic effect may be due to its caffeine, tanin, polyphenols and catechins allelochemical. The possible candidates interacted on the radicle of lettuce seedling and might inhibit radicle and hypocotyl elongation, total activity method will be used to investigate the induction of allelopathic activity. Our research aimed to determine the content of caffeine in tea samples and estimate its contribution to the inhibitory activity of tea samples.

4.2. Materials and Methods

Extraction

Dried tea leaves were ground into small pieces (about 1 mm) using a mortar and pestle, and 100 mg of ground tea sample was soaked in 100 ml of distilled water for 24 h at room temperature to analyze caffeine concentration by HPLC. The aqueous extract was filtered using a filter paper No.1 (ϕ150 mm, 0.2 mm thickness, Advantec Toyo Roshi Kaisha, Japan).

High performance liquid chromatography analysis (HPLC)

HPLC analyses of filtrates were carried out using an LC-20AD liquid chromatography provided by a shim-pack VP-ODS column (250 × 4.6 mm, 5 µm particles, Shimadzu). The oven temperature was kept at 40°C, and the flow rate was set at 0.35 ml min^{-1}. The gradient used was 0–20 min methanol/water (40:60, v/v), and before the injection (20 µl), the crude extract was filtered through a 0.2 µm syringe filter (Millipore). An SPD-M20A detector was used for the monitored analysis at 272 nm. The analyses were done in three replicates, and the results were calculated using a standard curve of pure caffeine.

Specific and total inhibitory activity bioassay

The specific and total inhibitory activity of the crude extracts and pure caffeine were estimated using lettuce seedlings as described by Golisz *et al.* (2007). The EC_{50} values were determined by a logistic regression equation of the concentration¬–response curves A filter paper (ϕ27 mm, Advantec Tokyo) was placed in a glass Petri dish (ϕ30 mm). For pure caffeine, 10 mg of pure powder caffeine was extracted with 10 ml distilled water; after stirring for 10 minutes, nine pure caffeine concentrations were diluted by distilled water into 0, 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, and 0.24 mg/ml; 700 µl of each diluted solution was added to the filter paper. Then, five lettuce seeds were placed on the filter paper and incubated in the dark condition at 22 °C for three days. Each treatment was replicated three times. The inhibition of lettuce radicle and hypocotyl elongation was evaluated by measuring the length and comparing them with that of the control. For crude extract, distilled water was used to extract the crude compounds from tea samples; 10 g of ground dried tea sample was extracted with 100 ml water for 24 hours, and the extracts were diluted (0, 0.5, 1, 2, 5, 10, and 15 mg/ml). The inhibitory activities were carried out by the same approach as pure caffeine (Fig 4.3).

The following formula was used to calculate the total activity of caffeine in all tea samples:

Total activity= caffeine concentration in tea leaves /specific activity of pure caffeine (EC_{50}) (1)

As the concentrations of the compound and the specific activity have the same dimension, the total activity is without any unit.

4.3. Results

4.3.1. Determination of caffeine concentration in tea samples

The concentration of caffeine from tea samples was found to be in the range of 20.7 to 38.2 mg/ml (Table 4.1). In the group of green tea samples, caffeine concentration ranged from 26.0–38.2 mg/ml; in oolong tea 21.4–23.3 mg/ml, 26.1 mg/ml in black tea, and 20.7 mg/ml in dried tea.

4.3.2. Specific and total inhibitory activity

Specific activity

The role of caffeine as an inhibitory compound in tea samples was clarified by the comparison between the inhibitory effect of each crude tea extract and pure caffeine on the radicle and hypocotyl of lettuce seedlings. Figure 4.1 shows the inhibitory effect of pure caffeine (at the same concentration obtained in the crude extract) and crude extracts of V1, V2, V3, V4, V5, V6 and V7 on the radicle growth of lettuce seedlings. The inhibitory effects of the pure caffeine and that of the aqueous extract of each tea samples on the radicle growth of lettuce seedlings were compared to determine the contribution of the caffeine into the total inhibitory activity of crude tea extract, and then choose the best treatment for tea allelopathy.

The inhibitory effect of pure caffeine on radicle growth was calculated by plotting the pure caffeine concentrations and its corresponding radicle growth. Based on the concentration of caffeine of each tea sample in the HPLC result and the linear regression equation, the actual concentration of the caffeine calculated to be present in the crude extract will then give the equivalent radicle growth. The inhibition effect of the caffeine in the extract is obtained. Figure 4.1 reflects the inhibitory activity curve of V2, V4, V5, V6 and pure caffeine has similar shapes of curves than that of V1, V3 and V7. Thus, caffeine accounts for most of the inhibitory activity caused by the crude tea extract of V2, V4, V5 and V6, which belong to groups of green and oolong tea samples, respectively. Additionally, the above result also can be confirmed by EC_{50} values of each crude tea extract and pure caffeine. In detail, the EC_{50} values of crude tea extract of V1 and V7 were 10.19 mg/ml and 10.16 mg/ml while the EC_{50} values of crude tea extract of V2 and V4 were obtained to be 1.22 mg/ml and 1.98 mg/ml, respectively. Compared with the EC_{50} value of pure caffeine estimated in tea crude extract, caffeine EC_{50} values were 2.44 and 3.48 mg/ml in V2 and V4, respectively.

Total inhibitory activity

A compound with the high total activity does not necessarily have a high specific activity (low EC_{50}) because the value of a total activity is a function of both the content of the compound and its specific activity. The total activity is a useful indicator to reveal the allelopathic potential of a compound (Fujii and Hiradate 2005). Based on the result of HPLC analysis, from Equation (1) the total activity of caffeine in V2 sample determined at 510, resulting in the highest activity compared to the other tea samples (270 to 510) due to its greater total activity value (Table 4.1).

4.4. Discussion

This analysis showed that green tea had higher caffeine concentration, and V2 contains the highest caffeine content among green tea samples. The difference in caffeine concentration in tea samples may be related to the season of tea harvesting, variety, and the manner of processing the material (Komes *et al*., 2009). The high caffeine concentration found in the green, oolong, and black tea may be due to the contribution of industrial processing, which is lacking in fresh tea. During the rolling stage in the manufacturing process, the cell wall of leaves is broken. This process makes the caffeine easily extracted by the solvent, resulting in higher caffeine concentration. Our result corresponded with the previous investigation by (Khoa *et al*., 2013) in which the caffeine concentration in fresh tea leaves ranged from 2.06% to 4.68% in dry matter. According to Ashihara and Kubota (1986), caffeine biosynthesis occurs in young tea leaves and buds. In this study, the V2 tea sample (mainly processed from the youngest tea bud) had the highest caffeine concentration. Moreover, in the production of V2, tea leaves are steamed immediately after harvesting and enzymes are inactive at the initial stage. Therefore, the decomposition of caffeine in processing steps may not happen (Le Thi *et al*., 2008).

Also, the caffeine concentration found in black tea was higher than that of oolong tea. The high amount of caffeine in black tea can be explained by the role of the fermentation process, which can slightly increase the concentration of caffeine. Stach and Schmitz (2001) found that during full fermentation of black tea, caffeine can combine with the polyphenols due to oxidation. Thus, caffeine was highly produced as the complex in storage and extraction. The results in Table 4.1 suggest that the alters in the phytotoxic potential may depend on the caffeine concentration. Tea samples with relatively high caffeine concentration had low specific activity and hence high phytotoxic potential. The release of caffeine from tea leaves significantly reduced the growth of lettuce seedlings in the Sandwich bioassay.

Almost all the values considered are closer to the EC_{50} value of the crude tea extract than that of V1, V3 and V7. From this information, caffeine may be the main contributor to the inhibitory activity of V2, V4, V5 and V6. Similarly, the result of Takemura *et al*., 2013 indicated coumarin was responsible for the inhibitory activity of leaves of *Gliricidia sepium* (Jacq.) Kunth when based on EC_{50} value of authentic coumarin and in the crude extract, respectively. We assume that other compounds such as polyphenols and catechins may also contribute to the inhibitory activity of V1, V3 and V7 samples. Moreover, this result was also similar to the inhibitory activity of caffeine on the hypocotyl growth caused by a crude extract of Vinatea-green tea and Thai Binh-Lang Son-oolong tea samples (Fig 4.2).

The total activity value of caffeine in V2 in this study was higher than the allelochemicals Momilactone B (0.01–0.1), DIMBOA (0.1), and (+)-2-cis-4-trans-ABA (0.07) (Fujii and Hiradate, 2005). Therefore, Vinatea-green tea (V2) can be considered as a potential material with the inhibitory substance being caffeine.

4.5. Conclusion

The results showed that the content of caffeine in group of green teas was higher than that of oolong teas and black teas. The inhibitory effect of these tea extracts was correlated to the amount of caffeine. The findings also indicated that caffeine accounted for a major part of the inhibitory activity of green tea and oolong tea. Caffeine inhibited the radicle growth of lettuce to 50% of the control at 75 μg/ml but caffeine was less effective on hypocotyl growth. The total activity of caffeine in Vinatea-green tea (V2) was considered a significantly total activity compared to allelochemicals of other reported allelopathic plants.

4.6. Tables and Figures

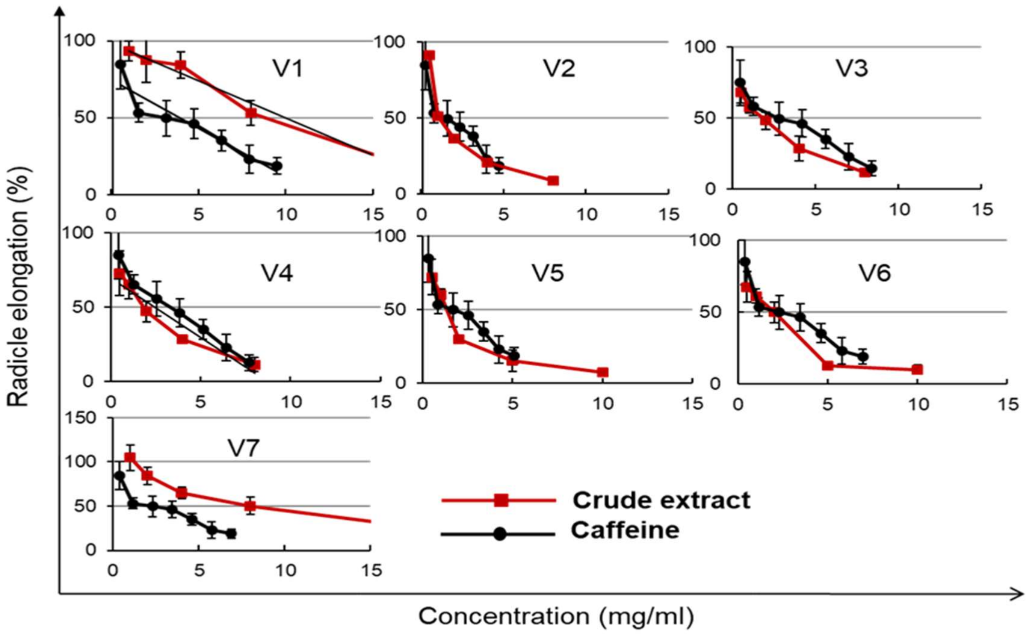


Figure 4.1. Inhibition activity of the radicle growth of lettuce seedlings caused by the crude extract of tea and by caffeine estimated to be present in crude tea extract

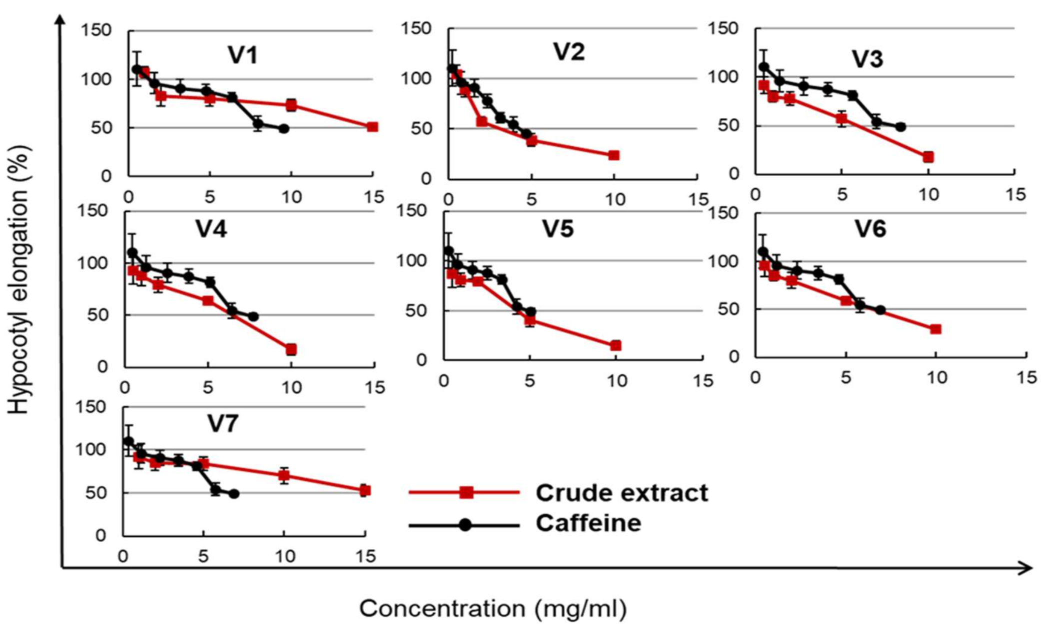


Figure 4.2. Inhibition activity of the hypocotyl growth of lettuce seedlings caused by the crude extract of tea and by caffeine estimated to be present in crude tea extract

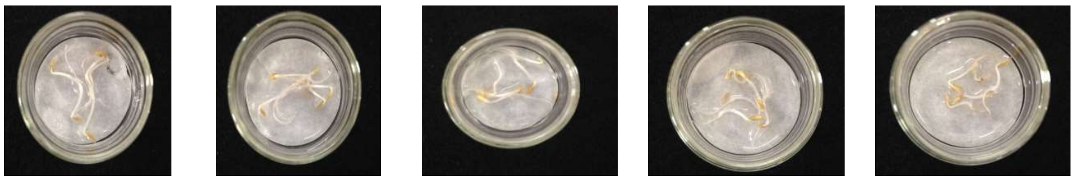
Table 4.1. Total activity of tea sample allelochemical (caffeine) on a plant basis

Samples ID	Type of tea	EC_{50} (mg D.W. per ml of water)	Concentration of caffeine (mg/ml)	Total activity (no unit)
V1	Fresh tea	10.2^{a}	20.7^{f} (±0.02)	270
V2	Green tea	1.22^{b}	38.2^{a} (±0.06)	510
V3	Oolong tea	1.31^{b}	21.4^{e} (±0.06)	290
V4	Oolong tea	1.98^{b}	23.3^{d} (±0.24)	310
V5	Green tea	1.56^{b}	35.5^{b} (±0.23)	470
V6	Green tea	2.10^{b}	26.0^{c} (±0.15)	350
V7	Black tea	10.2^{a}	26.1^{c} (±0.02)	350

EC_{50} of pure caffeine: 75 µg /ml. Values with the different common letter are significantly different ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replication ± SD.

Control

Pure caffeine



60 ppm 90 ppm 120 ppm 150 ppm 180 ppm

Green tea

1 mg/ml 2 mg/ml 5 mg/ml 10 mg/m

Back tea

2 mg/ml 5 mg/ml 10 mg/ml 15 mg/ml

Figure 4.3. The effect of concentration of pure caffeine and tea extract on the growth of lettuce seeds

CHAPTER V. EVALUATION OF ALLELOPATHIC POTENTIAL OF CAFFEINE FROM VIETNAMESE TEA ON GERMINATION AND GROWTH OF CROPS AND WEEDS

5.1. Introduction

Allelochemicals are present in several parts of plants that are known to interfere with seed germination and growth of neighboring plants by releasing allelochemicals in their environment (Sing *et al*., 2003; Rice *et al*., 1984).

The caffeine was also evaluated as the main contributor to allelopathic activity of green and oolong tea. However, each kind of weeds or crops has different sensitivity to inhibitory compounds extracted from allelopathic plants. The abundant investigation of allelochemicals selected for controlling weeds plays an important role in application in agriculture.

In this study, we evaluated the effect of aqueous tea extracts of Vinatea-green tea (V2) on the germination and growth of different species of plants and the effect of pure caffeine on the growth of radicle of some weeds and crops.

5.2. Materials and Methods

The following test plants were used to assess the possible phytotoxic effect of tea leaves extracts: Dicotyledonous species were: Lettuce (*Lactuca sativa* L. var. Great Lakes No. 366), white clover (*Trifolium repens* L.), red clover (*Trifolium pretense* L.), bird's–foot trefoil (*Lotus corniculatus* L.), hairy vetch (*Vicia villosa* L.), carrot (*Daucus carota* L.). Monocotyledonous species were: Italian ryegrass (*Lolium multiflorum* Lam.), perennial ryegrass (*Lolium perenne* L.), Timothy (*Phleum pratensis* L.), orchard grass (*Dactylis glomerata* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.) cv. Jhona and Shinshu. Parameters and germination days of sample seeds were presented in Table 5.1.

Table 5.1. Parameters and germination days of sample seeds

Plants	1000 seeds (gram)	Length (mm)	Height (mm)	Thickness (mm)	Germination (n- day)
Lactuca sativa	1.06 (±0.2)	3.16 (±0.29)	0.81(±0.09)	0.42 (±0.1)	10
Lolium multiflorum	2.61 (±0.12)	4.87 (±0.59)	1.23 (±0.14)	0.65 (±0.15)	10
Lolium perenne	1.97 (±0.08)	4.95 (±0.5)	1.09 (±0.11)	0.67 (±0.12)	10
Phleum pratensis	0.54 (±0.01)	1.55 (±0.26)	0.77 (±0.09)	0.73 (±0.12)	10
Dactylis glomerata	1.55 (±0.09)	6.33 (±0.48)	1.85 (±0.33)	0.65 (±0.16)	14
Trifolium pretense	2.23 (±0.03)	2.07 (±0.24)	1.43 (±0.13)	1.03 (±0.1)	7
Trifolium repens	0.60 (±0.02)	0.86 (±0.16)	-	-	7
Lotus corniculatus	1.41(±0.03)	1.20 (±0.1)	-	-	10
Vicia villosa	24.35 (±1.75)	3.35 (±0.28)	-	-	3
Daucus carota	1.48 (± 0.03)	3.09 (± 0.4)	1.33 (±0.15)	0.54 (±0.11)	4
Avena sativa	27.0 (±1.23)	10.66 (±1.03)	2.77 (±0.24)	2.21 (±0.26)	7
Hordeum vulgare	42.35 (±1.17)	8.31(±0.59)	3.53 (±0.18)	2.66 (±0.21)	7
Oryza sativa, cv. shinshu	25 (±0.04)	7.13 (±0.39)	3.15 (±0.15)	2.25 (±0.16)	10
Oryza sativa, cv. jhona	25.5 (±0.24)	8.98 (±0.32)	2.56 (±0.17)	1.95 (±0.07)	10

Extraction Procedure

The dried tea sample of Vinatea-green tea (V2) was ground into small pieces (about 1–2 mm) using the mortar and pestle, and 10 g of ground tea sample was extracted with 100 ml distilled water for 24 h. The crude extracts were then filtered twice using filter paper No.1.

Germination Bioassay

One milliliter of each extract (the final concentrations including 0, 50, 75, and 100 mg/ml) and treatment with water as the control was added to a sheet of 4.25 cm diameter filter paper in a 5.0 cm Petri dish. Ten seeds of each test species were placed on the filter paper and incubated in dark condition at 22 °C for n-days depending on plant species. Seeds that showed the emergence of the radicle were considered to have germinated. The number of seeds germinating was counted daily up to the final day (constant number of germinated seeds), and the percentage of germination was calculated by the formula:

$$\text{Germination (\%)} = (n/t) \times 100 \qquad (2)$$

where n is the number of seeds germinated and t is the total number of seed. Each treatment was replicated three times.

Seedling Growth Bioassay

A total of 700 µl of the crude extracts of tea leaves at the final concentrations of 0, 1, 2, 4, 6, 8, and 10 mg/ml were added to the filter paper. Five seedlings each of the test plants were put on the filter paper and incubated in the dark at 22 °C for three days. The length of radicle and hypocotyl/coleoptiles of test plants were measured on the third day. Each treatment was replicated three times. Treatment with water was used as the control. The working concentrations were different for lettuce seedlings than for weeds and crops because test concentrations for lettuce seedlings were done with all seven tea samples, while for weeds and crops, only Vinatea-green tea (V2) was used because it showed the highest inhibitory activity. Moreover, lettuce seeds are also sensitive to allelochemical and are mostly used as a reference in phytotoxic studies to determine any potential inhibitory activity. Concentrations of the extracts were different for germination bioassay than for seedling growth bioassay. Because the germination rate is not as sensitive as the growth rate of weed and crops, we made a range of higher concentration.

Ten milligrams of pure powder caffeine were extracted with 10 ml distilled water, and after stirring for 10 minutes, six pure caffeine concentrations were diluted by distilled water into 0, 0.05, 0.1, 0.15, 0.2, and 0.25 mg/ml concentration.

5.3. Results

Effect of Vinatea-green tea extracts and caffeine on the germination and growth of some crop and weed species.

The present study showed that the use of Vinatea-green tea extract negatively affected seed germination of all crops and weed at a concentration 100 mg/ml. However, at the concentrations of 50 mg/ml, the germination of some plants (oat, rice, red clover, timothy, hairy vetch, Italian ryegrass) was not significantly affected as compared to control. The results showed that the germination percentage of the test species depended on the concentration of tea extract.

The effect of Vinatea-green tea extract and pure caffeine on the radicle growth of crops and weeds is summarized in Table 5.1. The low EC_{50} value (1.24 and 1.12 mg/mL, respectively) of tea extract on timothy and white clover revealed their high sensitivity to tea extract. Also based on EC_{50} value, caffeine strongly suppressed timothy, white clover, red clover, barley, and birds-foot trefoil, and moderately inhibited the radicle growth of perennial ryegrass, Italian ryegrass, orchard grass, and oat. In contrast, caffeine had a slight effect on the radicle growth of carrot, hairy vetch, and rice seedlings.

5.4. Discussion

The effect on germination percentage by phytotoxic plants extracts in a dose-dependent manner was also reported in previous studies (Kato-Noguchi, 2001; Bogatek *et al.*, 2006). Nonetheless, Jhona rice cultivars were found to be highly resistant to tea extract at all tested concentrations, and the germination percentage was affected at a very high concentration of 100 mg/ml (Fig 5.1). Besides tea extract, caffeine from seed of *Coffea arabica* L. completely delayed germination of the test weeds such as *Amaranthus spinosus* L., *Echinochloa colonu* L., *Echinochloa crus-galli* L., and *Vicia sativa* L. at 1200, 2000, 2500, and 10,000 µg/ml; similar treatments of the seeds of *Phaseolus mungo* L. observed little effect (Rizvi *et al.*, 1981). Khursheed *et al.*, 2009 indicated that caffeine at lower concentrations had a stimulatory effect (probably act as growth regulator) on growth and yield in *Helianthus* *annuus* L. However, higher doses reduced the growth and yield. The current result also clearly demonstrated that caffeine had little effect on crop plant, as it selectively inhibited the weeds species. This selective inhibition is similar to L-3,4-dihydroxyphenylalanine allelochemical from *Mucuna pruriens* (L.) (Fujii *et al*., 1991). From the present preliminary investigation, it can be concluded that green tea leaves exhibited remarkable phytotoxic potential by significantly inhibition of the germination and radicle growth of test plants; it is also possible to apply the tea extract for weed management directly by using proper concentration.

It was also indicated that the hypocotyl or coleoptile growth of crops and weeds seedling was less sensitive to the tea extracts and caffeine, compared to radicle. The radicle is the first organ to absorb allelochemicals from extract solution and is required for both cell expansion and cell proliferation. Thus, the permeability of allelochemicals into radicle tissue is higher than hypocotyl or coleoptile tissue (Nishida *et al*., 2005). The observed difference in response of the test plants (germination, radicle, and hypocotyl/coleoptile elongation) to tea extracts and caffeine is possibly due to the differences in seed size and seed coat permeability, which responsible are for caffeine uptake, or the seed anatomy, germination duration, and nature of the test species (Hassan *et al*., 2012). One of the suggested reasons is that inhibition of germination by caffeine causes a marked decrease in amylase activity (Rizvi *et al*., 1987).

The present result showed Vinatea-green tea contained caffeine, which possesses inhibitory activity against some weeds species and, thus, it may be used as a potential material in sustainable weed management. However, for oolong, black, and fresh tea, we cannot conclude that they are not useful in this study, since some other research also indicated that fresh and black tea extract significantly inhibited the germination and growth of garden cress (*Lepidium sativum* L.), lettuce redroot pigweed (*Amaranthus retroflexus* L.), and golden foxtail (*Setaria glauca* L.) at specific concentrations. Moreover, black tea residue extracts suppressed wheat and maize seed germination and growth, while methanol extract completely inhibited seed germination (Rezaeinodehi *et al*., 2006; Waris *et al*., 2016).

5.5. Conclusion

Aqueous extract of tea sample (V2) significantly affected the germination and especially the radicle growth of timothy, white clover. Also, this may be attributable to the fact that roots have direct contact and are the first to come in contact with allelochemicals. The phytotoxicity of extracts was strongly increased as their concentration increased, caffeine exerted a differential action on several plant species, this selectivity resembles other candidates of allelochemicals, and caffeine might be a useful selective herbicide.

5.6. Table and Fugures

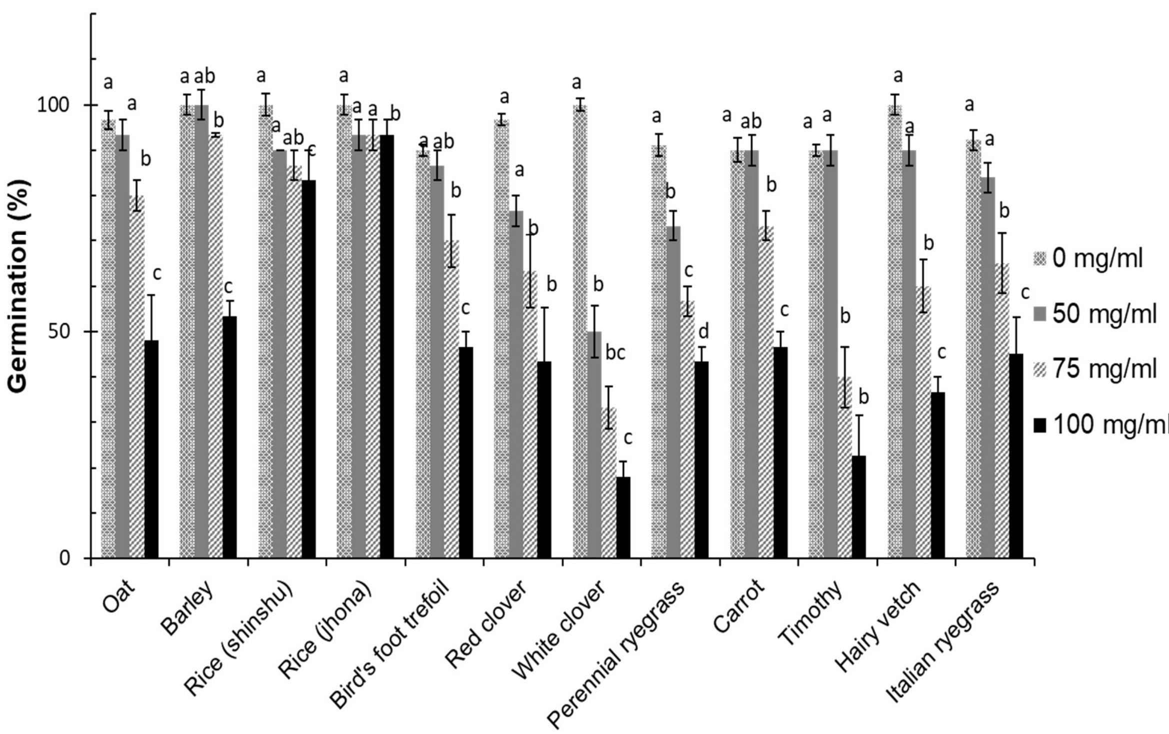


Figure 5.1. The effect of Vinatea-green tea extract on the germination of crops and weeds

Columns followed by the same letter, within the same crop or weed, are not significantly different ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replications ± SD.

Table 5.2. Effect of Vinatea-green tea extraction and caffeine on radicle growth of the crop and selected weed species

Scientific name (English name)	EC_{50} (mg/ml)[a]	
	Crude extract	Pure caffeine
Phleum pratensis	1.24[a]	0.15[ab]
Trifolium repens	1.12[a]	0.10[a]
Trifolium pretense	3.01[ab]	0.10[a]
Hordeum vulgare	4.34[bc]	0.15[ab]
Lotus corniculatus	5.06[c]	0.10[a]
Lolium perenne	5.62[c]	2.50[b]
Lolium multiflorum	5.17[c]	2.50[b]
Dactylis glomerata	5.91[c]	2.50[b]
Avena sativa	6.02[c]	2.50[b]
Vicia villosa	8.32[d]	>2.50[c]
Daucus carota	8.64[de]	>2.50[c]
Oryza sativa, cv. Jhona	8.82[de]	>2.50[c]
Oryza sativa, cv. shinshu	10.4[e]	>2.50[c]

[a]EC_{50} (mg/ml) is the concentration at which the radicle length became 50% of the control. Values with the different common letter are significantly different. ($p < 0.05$, Tukey and Dunnett test). >2.50 means EC_{50} are bigger than 2.50 mg/ml. The data are the mean of three replications ± SD.

CHAPTER VI. EVALUATION OF THE PHYTOTOCXIX ACTIVITY AND CONCENTRATION OF CAFFEINE FROM TEA RESIDUES IN THREE KINDS OF SOILS AND TEA GARDEN SOIL

6.1. Introduction

Exudation of allelochemicals from living plants was found in the water and agar cultures experiments. However, the detection of allelochemicals in the soil of a growing donor plant is necessary to recognize allelopathy under field conditions. The role of soil is very important in the evaluation of allelochemicals potential of agricultural ecosystems, while the concentration of allelochemicals is affected significantly by the presence of soil (Norouzi *et al*., 2015). Oleszek and Jurzysta (1987) mentioned the influence of soil texture on phytotoxic effects. Inderjit and Dakshini (1994) found that the amounts of water-soluble phenolics in *P. lanceolata* leaf leachate amended soil varied depending on the soil textural classes (clay, sandy-loam, sand, and silty-loam).

Moreover, in agricultural cultivation, autotoxicity usually results in a decrease of crop yield and quality. Root exudates from plants influence the growth of other plants through soils, which is one of the pathways of allelopathy. Caffeine is present to the greatest extent in the young leaves of tea, its accumulation in old tea plantations might result in tea autotoxicity, and young roots of tea are highly susceptible to caffeine (Anaya *et al*., 2002). Also, it is suggested the concentration of an allelochemical in soil is a dominant factor directly determining the phytotoxic activity in soil, and the concentration is controlled by soil factors that affect the behavior of adsorption, desorption, and degradation in soil. Laboratory studies on the functions of isolated chemicals, no matter how mechanistically detailed, cannot demonstrate the significance of allelopathy in communities. Evidence for allelopathy in natural plant communities should include information of concentrations and release rates such as demonstrated in field soils for caffeine

In order to study phytotoxic of caffeine in soil, three approaches are followed; the effect of soil types (Fluvisol, Andosol, Sand) on possible phytotoxic of caffeine from tea residues on the growth of lettuce seedling, rhizosphere soil method and the actual concentration of caffeine in soil farm of tea

6.2. Materials and Methods

The Andosol soil was collected from the experimental farm of Tokyo University of Agriculture and Technology and Fluvisol soil was taken from Field Museum Fuchu Honmachi, Field Science Center, Tokyo University of Agriculture and Technology. Sand soil was taken from Fujii Laboratory, Tokyo University of Agriculture and Technology.

Table 6.1. Physic - chemical properties of experimental soils (Shwe. 2015)

Properties	Andosol	Fluvisol
Clay (%)	2.5	15.1
Silt (%)	74.3	46.8
Sand (%)	23.2	38.1
pH	5.9	6.1
Dissolved organic carbon (mg kg^{-1})	4.9	10.7
Soil type	**Silt loam soil**	**Loamy soil**
NH_4-N (mg kg^{-1})	0.2	0.6
NO_3-N (mg kg^{-1})	1.2	2.6
Total nitrogen (g kg^{-1})	4.5	3.7
Total carbon (g kg^{-1})	67.3	38.3

For Sand soil: 77.8 (%) Sand, 13.7(%) Silt, 8.5 (%) clay, 3.4 (%) organic matter, pH =8.1, 0.095 (mg/g) K, 0.2 (mg/g) Na, 1.6(mg/g) Ca, 1.85(mg/g) Mg, 1.04 (mg/g), $HC0_3$ (Salama *et al*., 2014)

Determination of the effect of type of soil on the content of caffeine from tea residues in the soil and its allelopathic activities.

10g of Andosol, Fluvisol, and Sand soils after autoclaved at 121ºC for 20 minutes and were placed in each well of the six-well multi-dish plastic plates. Tea leaves (50 and 100 mg) were mixed with soil and 3.5, 2 and 1.5 ml of water were added to 10g of Andosol, Fluvisol and Sand soil respectively. After 5 days of incubation (22ºC), 5g of each type of soil was used to analysis the caffeine content by HPLC and same amount of soil was used to test the allelopathic activity of soil.

3.5, 2, 1.5 ml of 1000 ppm pure caffeine water solution were added to 10g each of Andosol, Fluvisol and Sand soil respectively and then placed for incubation. After 5 days, 5g soil were extracted by pure methanol, 90% methanol and water for 24 hours and filtered, caffeine content was measured by using HPLC (the analysis condition is similar to caffeine analysis in tea samples as described above) and 5g other soil also was tested for the allelopathic activity (Fig 6.1).

All types of soils were sterilized, and tea samples were treated under UV light to avoid the effect of microorganisms.

Soil Sampling for Rhizosphere Soil Method

The samples from tea plants (Vinatea-green tea) were taken out from the tea farm (Fig 6.2). Then, soil adhering to the surroundings of the root "rhizosphere soil" and the soil shook off "root-zone soil" were collected. After that, collected soils were sieved in a 1 mm mesh removing root hair as much as possible. In brief, 5 ml of agar was added into the 6-well multi-dish containing 3.0 g of soil (in dry weight). On the surface of this agar, 3.2 ml of agar was over-layered, and lettuce seeds were put on this surface. Meanwhile, in control treatment, the same steps were followed, except that soil was taken from a place that had not been planted before with Vinatea-green tea with three replications for each treatment. After three days of incubation, the germination percentage and growth of lettuce seeds were measured (Fujii *et al*., 2005).

Analysis of Caffeine Residue in the Soil of tea garden

Soil samples were collected randomly from three locations inside the Vinatea-green tea in November 2017, within a depth of 10–15 cm using soil auger. From each location, we collected three samples; then, these three samples were mixed thoroughly together to obtain a homogenized representative sample. The soil samples were air-dried, and materials like roots, stones, and pebbles were removed. After crashing, soil sieved through a 2 mm sieve to get fine soil particles free of any plant materials. Then, 150 g soil was extracted two times with 300 ml of chloroform for 72 h by shaking (Fig 6.3)

The chloroform extract was evaporated and re-dissolved in 100% methanol. Methanol extracts were filtered through a 0.2 μm syringe filter for HPLC analysis. To ensure good data quality, the efficiency of the analytical method (the extraction and clean-up methods) was determined by the recoveries of an internal standard (standard caffeine). Clean soil sample (agriculture soil collected out of this farm) was spiked with a known amount of standard caffeine and extracted under the same conditions as Vinatea-green tea soil samples. After that, we determined what percentage of the caffeine applied was recovered by extraction and clean-up methods.

6.3. Results

6.3.1. Determination of the effect of type of soils on the content of caffeine from tea in the soil and its allelopathic activities.

When pure caffeine water solution was added to all types of soils, the caffeine content (746 ppm) was found to be higher with pure methanol than that of extraction with 90% methanol (690 ppm) and water (196 ppm) in Andosol soil, this result similar to Fluvisol and Sand soil. Pure methanol extraction had shown the highest recovery percentage of caffeine content on all types of soil and can be used as a best solvent to extract caffeine in soil (Fig 6.4). Among three soils extracted with pure methanol, the content of caffeine in Andosol soil was 746 ppm which was 2 times and 3 times as great as that found in Fluvisol (311 ppm) and Sand soil (254 ppm). So, it is clear that the recovery of caffeine content was high in the following order: Andosol>Fluvisol>Sand soil.

Result of the effect of pure caffeine on radicle and hypocotyl growth of lettuce seedling in 3 types of soil showed in (Fig 6.5). Caffeine had specific activity with an EC_{50} of 1000 ppm for radicle growth lettuce seeds was found in Andolsol soil.

Fluvisol soil strongly absorbed the caffeine. The result demonstrated that caffeine concentration recovery (169 ppm) in Fluvisol from green tea at a concentration (10 mg tea /g soil) was lowest, compared to Andosol and Sand soil (663 and 448 ppm respectively). The caffeine adsorption in soil depends on different tea types (adding to soil) and its concentration as well as the property of each type soils. The green tea sample added to all three soils was characterized by the highest caffeine concentration which was analyzed by HPLC in Andosol soil while Fluvisol soil the lowest (Fig 6.6)

The presence of soil decreased plant growth inhibitory activity by caffeine. In this study, the effect of caffeine on reducing of lettuce radicle growth was not observed when tea was applied in Fluvisol soil (due to low caffeine content in this soil). Based on EC_{50} (10mg green tea/g soil) value, could account for strongest inhibitory activity on radicle growth of lettuce seeds of Andosol soil and Sand soil (Fig 6.7).

The caffeine concentration in soil had a strong positive correlation with radicle growth inhibition of lettuce seeds (Fig 6.8)

6.3.2. Phytotoxic potential of caffeine from Vinatea-green tea in soil

To study the actual effect of caffeine in soil farm of Vinatea-green tea, rhizosphere soil and root-zone soil have been assessed on lettuce seeds. The results showed that the inhibitory on the growth of lettuce seedling by rhizosphere soils were stronger than those of root-zone soils. Rhizosphere soil under Vinatea-green tea plant inhibited the radicle elongation of lettuce seedling by 66% compared to control (Fig 6.9). Thus, compounds released by Vinatea-green tea plant roots and adsorbed into rhizosphere soil particles showed phytotoxic potential.

Also, to confirm the existence and availability of caffeine in the soil of V2, the concentration of caffeine has been measured by HPLC. The analytical results showed that caffeine concentration ranged from 0.137 to 0.145 μg/g soil (Table 6.2), and recovery and reproducibility for caffeine extraction from spiked soil were satisfactory (approximately 75%).

6.4. Discussion

The results showed that the concentration of caffeine in three soils decreased in the following order: Andosol>Sand soil >Fluvisol.

The recovery of caffeine or caffeine concentration in Fluvisol soil was lowest because Fluvisol soil (negative charged site), with highest clay content(15.1% clay), organic matter and moisture, as well as dissolved organic carbon (Shwe, 2015) might involve the effect of variations in clay content on adsorption and transformation reactions of caffeine, however, the mechanism was not further study yet, moreover, clay soil is poorly drained result in minimum toxin leaching, so caffeine concentration was analyzed to be lowest which had no effect on plant growth inhibition. Results of Furubayashi *et al*., (2005) also indicating L-DOPA adsorption and the influence of clay content on absorption and transformation reactions were not clear. In Sand soil, pH value high (8.1) and well-drained soil reduced caffeine concentration. In soils with high pH values, the plant-growth-inhibitory activity of L-DOPA was not detected (Furubayashi *et al*, 2005).

The recovery of caffeine in Andosol soil was highest, due to the lowest pH value and clay content compared to Fluvisol and Sand soil. Thus, caffeine adsorption and transformation reactions were difficult to happen, leading the effect of caffeine reduction in this soil was not significant. High caffeine concentration in Andosol soil had a strong positive relationship with plant growth inhibition.

This concentration was not so high and may be related to the physical properties of the caffeine molecule, which is soluble in water. So, it is easily leached by rain into groundwater. Detection of caffeine in soil indicated that caffeine could directly affect the growth of plants in soil, and the effect depends on concentration. Our result agrees with (Ito *et al*., 1998), who reported that the concentration of dehydromatricaria ester (DME) in soil water is low due to most of DME in the soil being either adsorbed to soil solids or degraded by microbes, and the phytotoxicity of DME was shown to depend on.

6.5. Conclusion

The phytotoxicity of allelochemicals affected by soil factors, the plant-growth-inhibitory activity of caffeine was markedly reduced when caffeine was applied on a Fluvisol. The phytotoxic activity was more noticeable in Andosol soil and Sand soil.

Allelopathic potentiality in rhizosphere soils suggests that caffeine released from roots of plants are adsorbed on soil. Caffeine accumulation in tea garden at different soil extracts, in the leaves of tea into the soil by rain, dew or fog and decomposition of tree residues. This accumulation leads to the inhibition of tea seed germination, seedling growth of neighboring of plant. Caffeine concentration was found relatively small concentrations, but it confirms the presence of caffeine in soil therefore finding out the possible selection methods of succeeding crops to avoid the allelopathic effects in the next crop are the ways for sustainable development in agro-ecosystem.

6.6. Tables and Figures

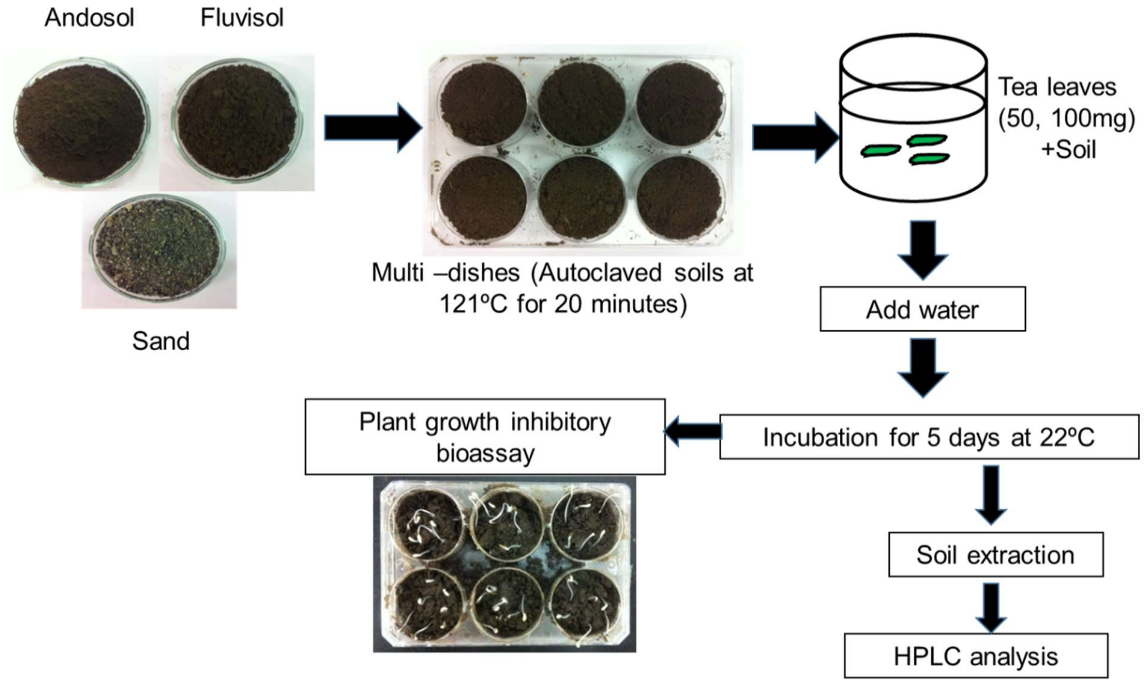


Figure 6.1. The incubation of tea leaves in soil procedure

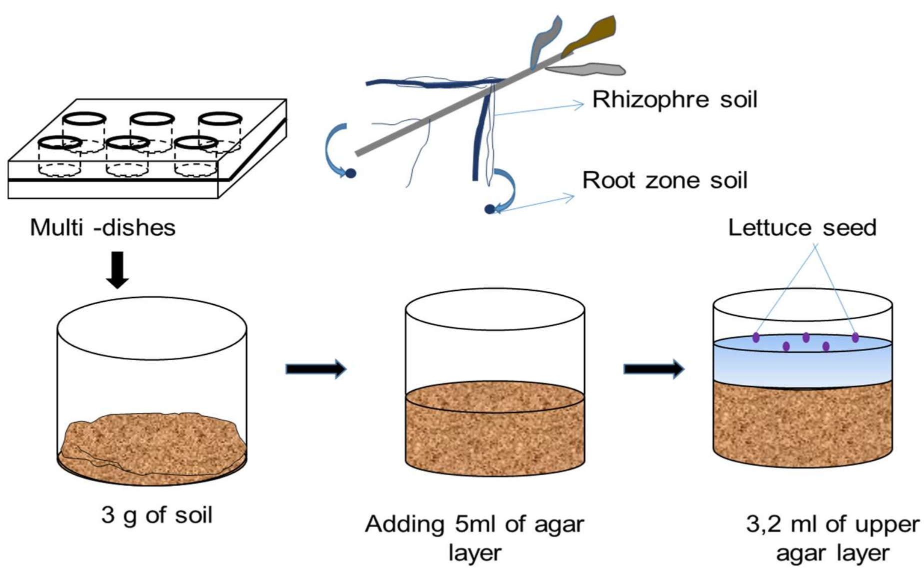


Figure 6.2. Rhizosphere soil method for evaluation of caffeine in soil (Fujii *et al*., 2005)

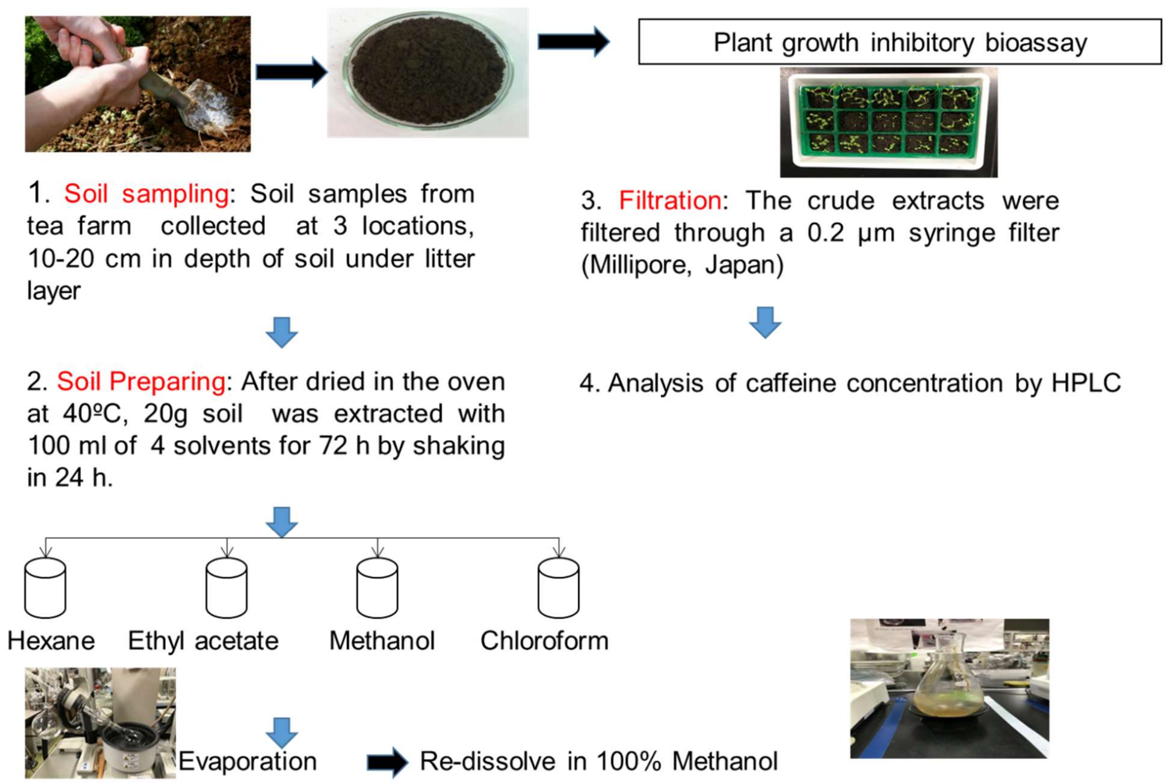


Figure 6.3. Assessment of allelopathic activity of caffeine accumulation in the soil, from tea garden

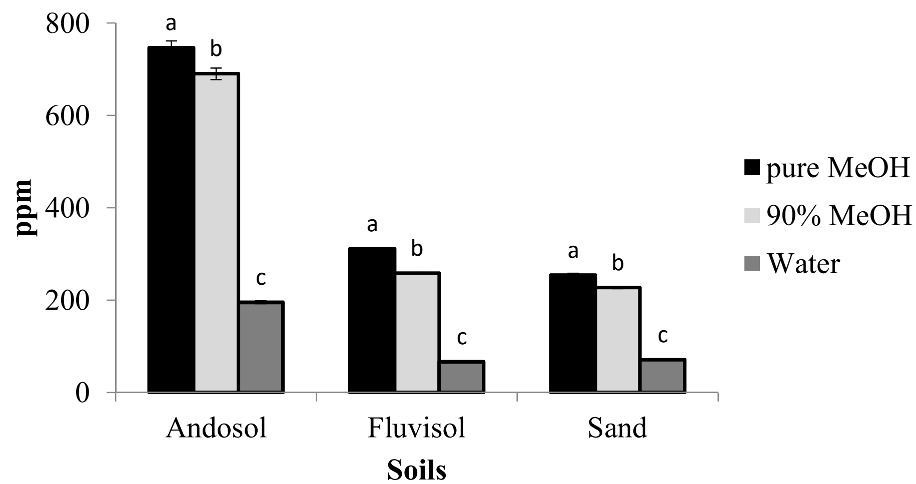


Figure 6.4. The effect of solvent and soil types on recovery of caffeine content

Statistical analysis method was performed to compare caffeine concentration induced by pure caffeine in three soils at three extraction solution group by group. Values with the different common letter are significantly different between each soil type. ($p < 0.05$, Tukey and Dunnett test).

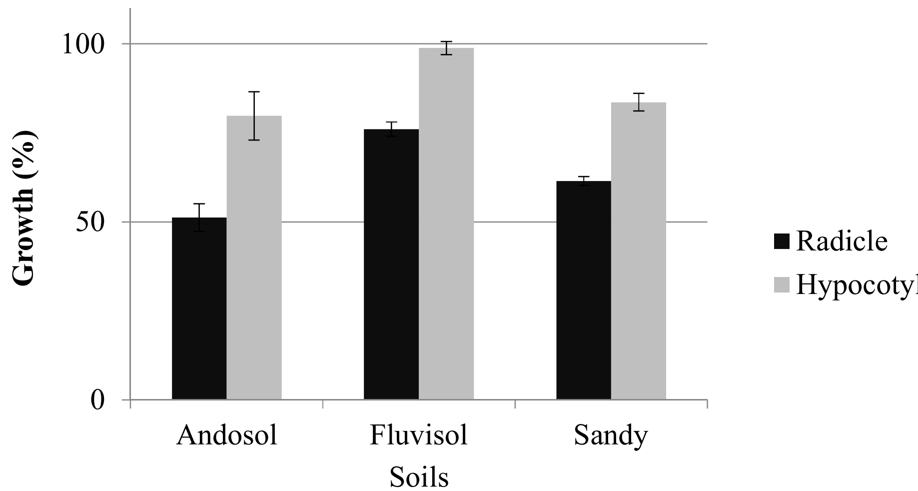


Figure 6.5. The effect of pure caffeine on Radicle and Hypocotyl growth of lettuce seeds in three types of soil

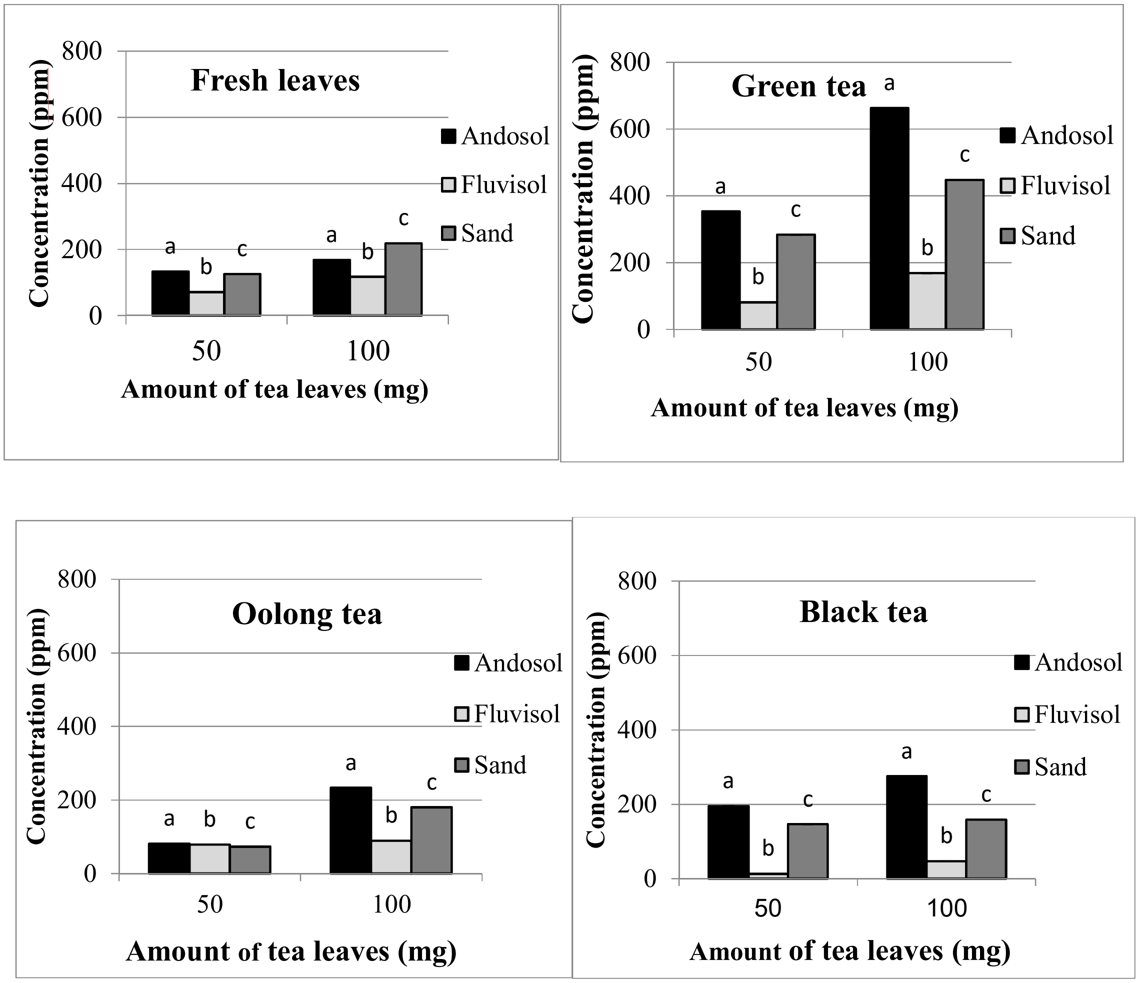


Figure 6.6. The adsorption of caffeine from tea leaves in different kind of soils

Statistical analysis method was performed to compare caffeine concentration induced by tea samples in soil at 50 or 100 concentrations group by group. Values with the different common letter are significantly different. ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replications ± SD.

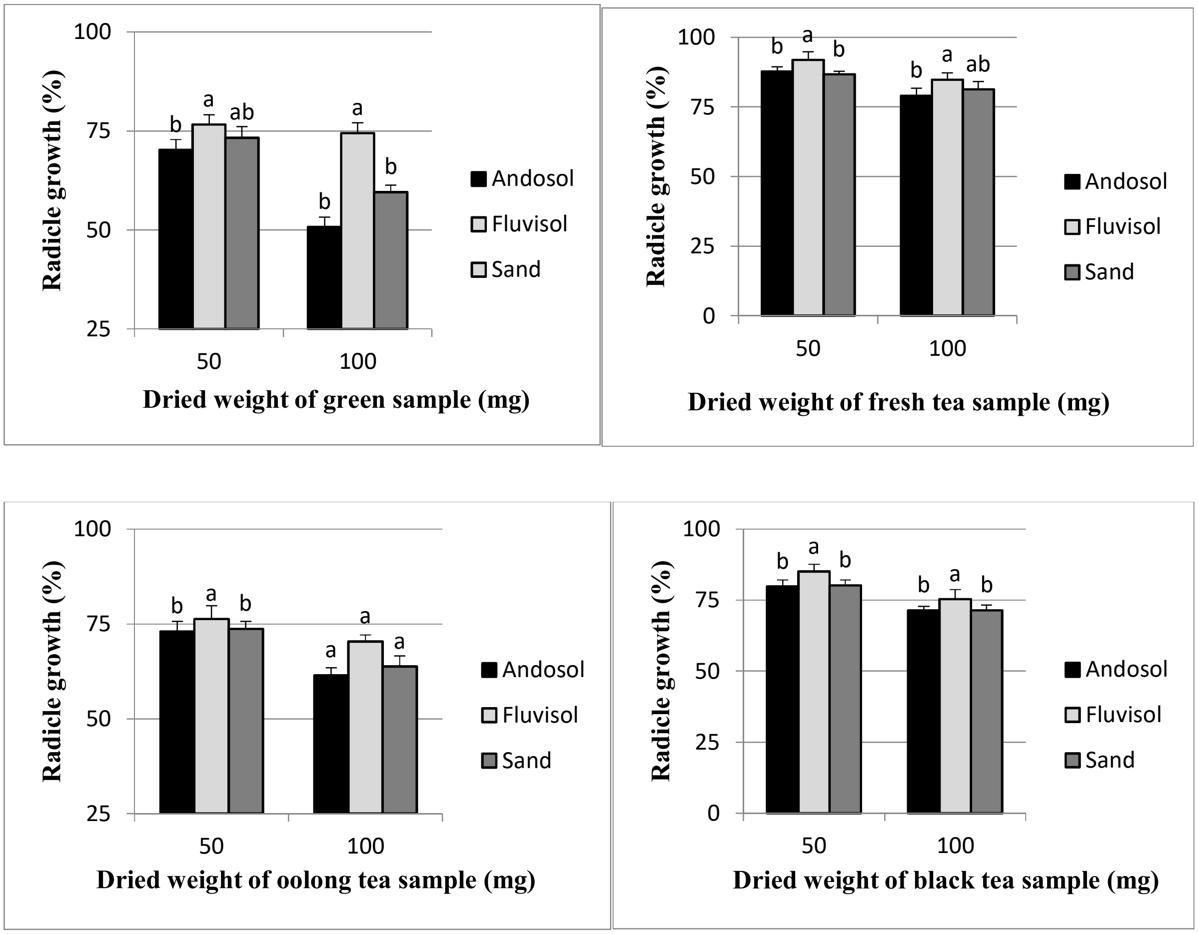


Figure 6.7. The effect of tea added to soil on the radicle growth of lettuce seeds

Statistical analysis method was performed to compare inhibition percentage induced by tea samples in soil at 50 or 100 concentrations group by group. Values with the different common letter are significantly different. ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replications ± SD.

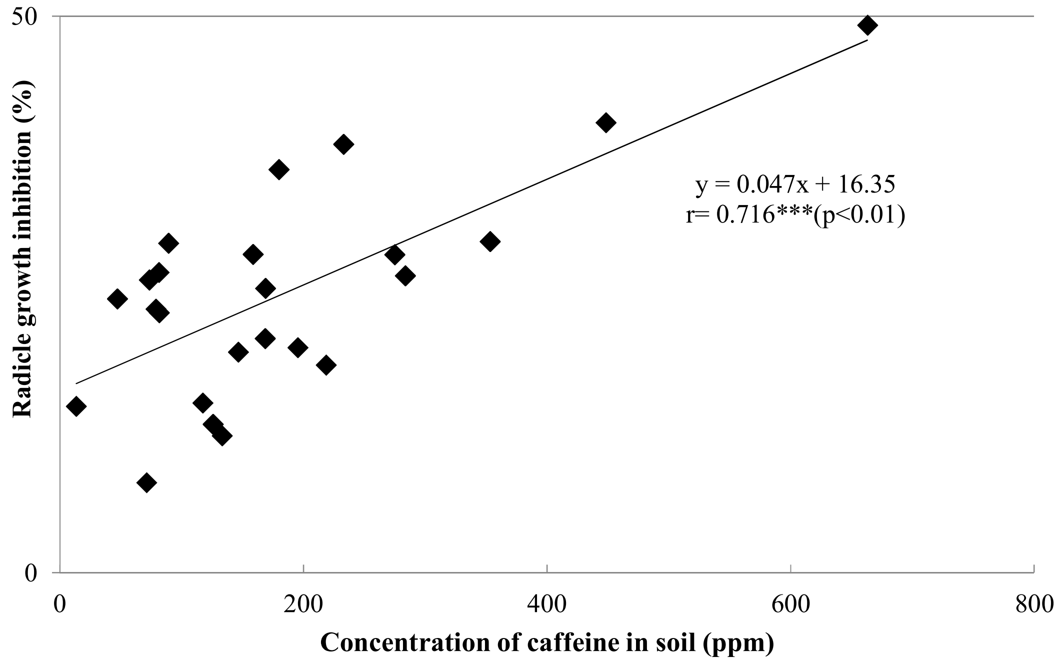


Figure 6.8. Relationship between the inhibition on radicle growth and concentration of caffeine in soils

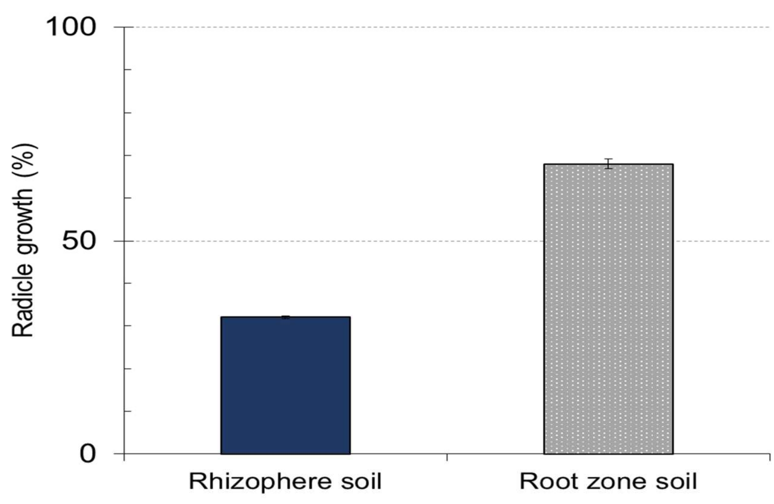


Figure 6.9. The effect of the phytotoxicity of rhizosphere soil and root-zone soil on the growth of lettuce seedlings by the rhizosphere soil method

Table 6.2. The concentration of caffeine from the soil collected from three different locations at Vinatea -green tea farm

Locations	Concentration of caffeine in soil (µg/g)
1	0.137 (±0.004)
2	0.142 (±0.002)
3	0.145 (±0.005)

Values are means of three replications ± standard deviation.

CHAPTER VII. ALLELOPATHIC EFFECT OF PUTATIVE ALLELOCHEMICAL (CAFFEINE) IN CALLUS OF TEA PLANT

7.1. Introduction

To evaluate the allelopathic activity at the cellular level, the recent protoplast co-culture method with digital image analysis was applied. The protoplasts were isolated from callus and were co-cultured with recipient lettuce protoplasts (Sasamoto *et al*., 2013). This method may be useful as a sensitive bioassay of allelopathy.

By using the same lettuce protoplast culture method, caffeine has also been quantitatively investigated when thirteen days of treatment with >250 µM, caffeine had a marked inhibitory effect on the colony formation of cells derived from the protoplasts. These observations suggest that a relatively low concentration of caffeine is toxic for the proliferation of plant cells (Sasamoto *et al*., 2015).

The green tea was considered to have allelopathic activity. In Japan, green tea – producing area of Yabukita, one of tea plan varieties account for approximately 76% of all tea producing area in this country (Kodama *et al*., 2007). In this present study we first screened the allelopathic effect of calluses induce from Japanese tea plant varieties and determine the content of caffeine in tea callus.

7.2. Materials and Methods

Callus cultures were established from leave of Sazanka tea (*Camellia sasanqua*), Tsubaki tea (*Camellia japonica*) and Yabukita tea (*Camellia sinensis*). Original calluses were provided by Prof. Ogita through Prof. Sasamoto, proliferated calluses were sub-cultured on the medium composition the Murashige and Skoog's (MS, Murashige & Skoog, 1962) MS basal medium containing 3% sucrose, 1 µM of 2,4-D and 0.1 µM of benzyladenine (BA), solidified with 0.8% agar (Fig 7.3).

Proliferated calluses were sub-cultured in the dark at 3- to 4-week intervals at 27 ºC and were used for examination of caffeine content by using HPLC and protoplast isolation or stored in a freezer at -80 ºC.

Sandwich Method

Fresh leave of Sazanka, Tsubaki and Yabukita tea were dried at 60 ºC overnight, fresh callus of 1.08 g was dried at 60 ºC for 5 hours. The procedure of the sandwich method was as described previously (Fujii *et al*., 2003) (Fig 7.4). The control treatment consisted of seeds germinated in the absence of dried leaves. Data were recorded as % growth of the control and averaged with standard deviation.

Extraction of Callus

0.51 g fresh callus was homogenized with 3 ml of 80% aqueous MeOH centrifuged at 4000 × *g* for 10 min. The supernatant was collected and 2 ml of 80% aqueous MeOH. The MeOH-soluble fractions (5 ml) were combined and evaporated to dryness. The resulting materials were dissolved in 1.5 ml distilled water HPLC.

HPLC analyses of filtrates were carried out using an LC-20AD liquid chromatography provided by a shim-pack VP-ODS column (250 × 4.6 mm, 5 µm particles, Shimadzu). The oven temperature was kept at 40 °C, and the flow rate was set at 0.35 ml min^{-1}. The gradient used was 0–25 min methanol/water (50:50, v/v), and before the injection (20 µl), the crude extract was filtered through a 0.2 µm syringe filter (Millipore). An SPD-M20A detector was used for the monitored analysis at 272 nm. The analyses were done in three replicates, and the results were calculated using a standard curve of pure caffeine.

7.3. Results

The results of the sandwich method demonstrated a strong inhibitory effect of Yabukita tea (by 90%) at concentration of 1 mg dried leave/ml agar while Tsubaki showed a weak inhibitory effect on root elongation of lettuce seedlings (Fig 7.1). Inhibitory effect of Sazanka in-between that of Yabukita tea and Tsubaki.

The callus also showed similar inhibitory activity. Root elongation of lettuce was slightly (75 %) inhibited by callus of Tsubaki, and strongly (80–85 %) inhibited by callus of

Sazanka and Yabukita tea (Fig 7.2). The inhibitory activity of Yabukita callus was among the strongest of allelopathic plants.

Figure. 7.6 shows the HPLC profile of caffeine in the calluses of tea. The peak at 13.28 min was the main peak of caffeine. The calculated content of the main peak was 19.21 µg/g callus. Compared to Sazanka and Tsubaki tea, only in Yabukita tea caffeine was identified. According to the results of (Li *et al*., 2008) HPLC analysis indicated that the major purine alkaloid in (*Camellia sinensis* cv. Yabukita) tea callus line is caffeine, some pigment –forming tea calluses line have caffeine biosynthesis capability.

7.4. Discussion

Yabukita tea showed allelopathic potential and contained caffeine as major compound, strongly inhibition in callus of Yabukita can be explained by the presence of caffeine however its existence at small amount. This agreement with the report of callus often have a lower content of metabolites than the original plant tissues, the content of the allelochemical L-DOPA was also lower in callus cultures than in the original plant tissues (Sasamoto *et al*., 2013). Higher inhibitory activity was observed in the young green leaves might due to the accumulation of allelochemicals during the late growth stage of plants (Sasamoto *et al*., 2012) compared to that of callus. Allelopathic effect of Sazanka and Tsubaki can be contributed by other compounds instead of caffeine.

7.5. Conclusion

As the same strong inhibition was obtained with by the sandwich method in both dried leave and callus of Yabukita, the mechanism is assumed to be the same caffeine was detected.

Inhibitory effects of protoplasts from tea on the recipient protoplasts of cotyledons of *Lactuca sativa* (lettuce) co-cultured using 96 multi-well culture plates were done in next step.

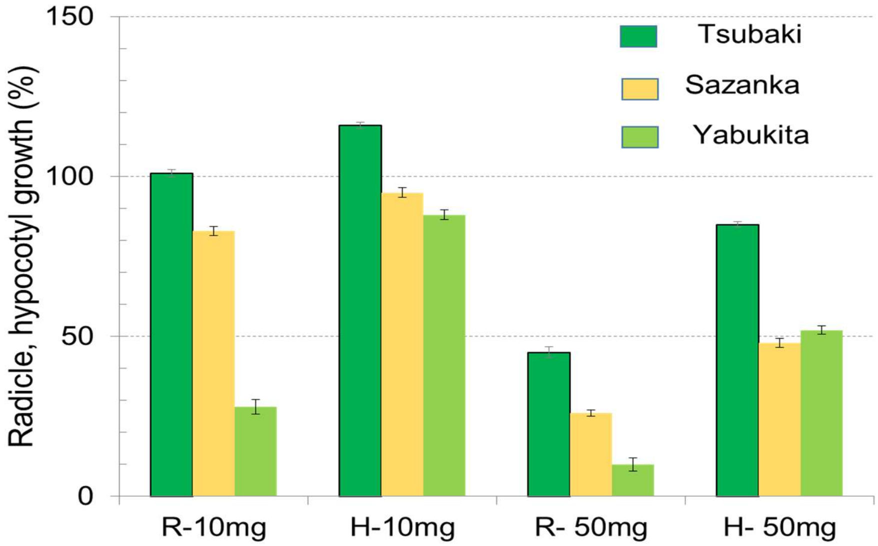


Figure 7.1. Effects of 10 and 50 mg of leaves of Tsubaki, Sazanka and Yabukita on hypocotyl and root elongation of lettuce

Data are average percentages of control with SD. hypocotyl (H). root (R)

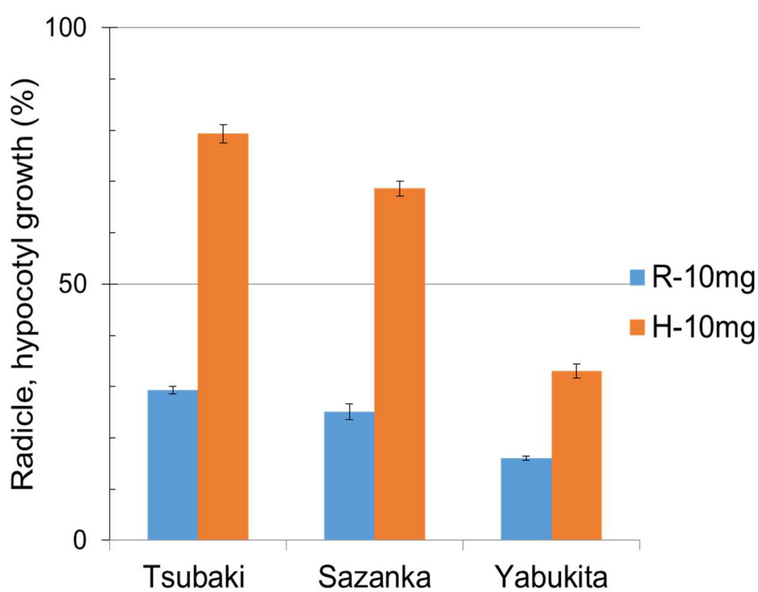


Figure 7.2. The effect of allelopathic activity of tea callus on the growth of lettuce seedling by Sandwich method

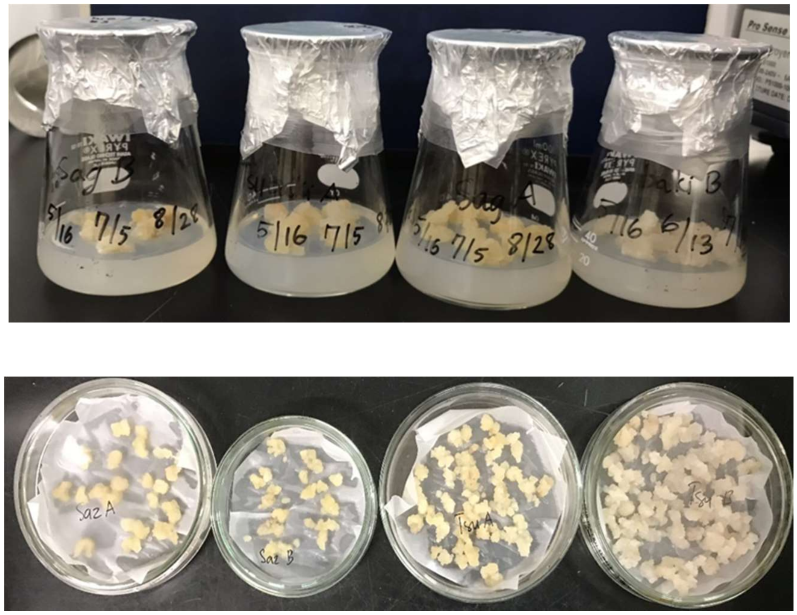
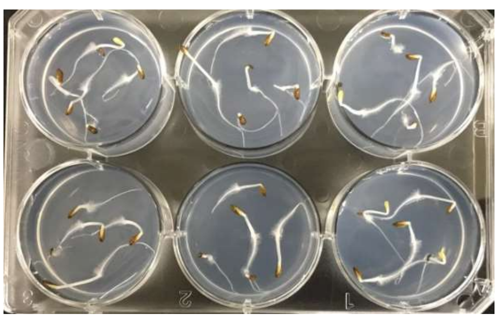
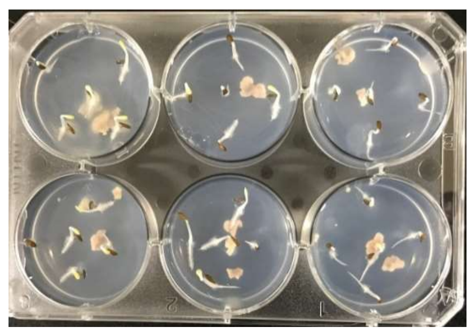


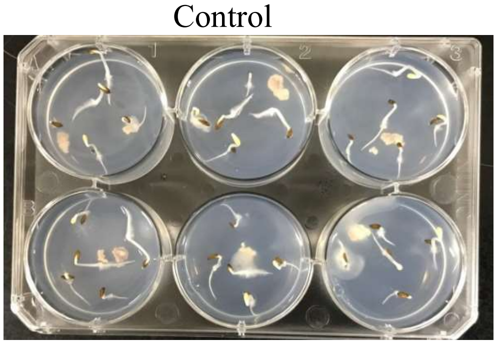
Figure 7.3. Calluses from tea



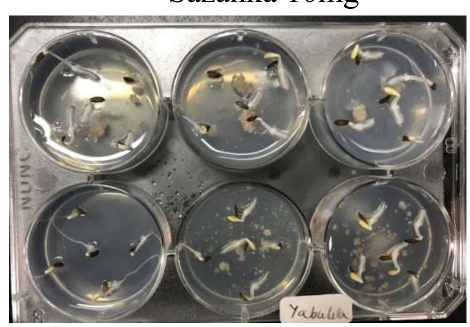
Control



Sazanka 10mg



Tsubaki 10mg



Yabukita tea 10mg

Figure7.4. The effect of allelopathic activity of calluses on the growth of lettuce seedling by using Sandwich method

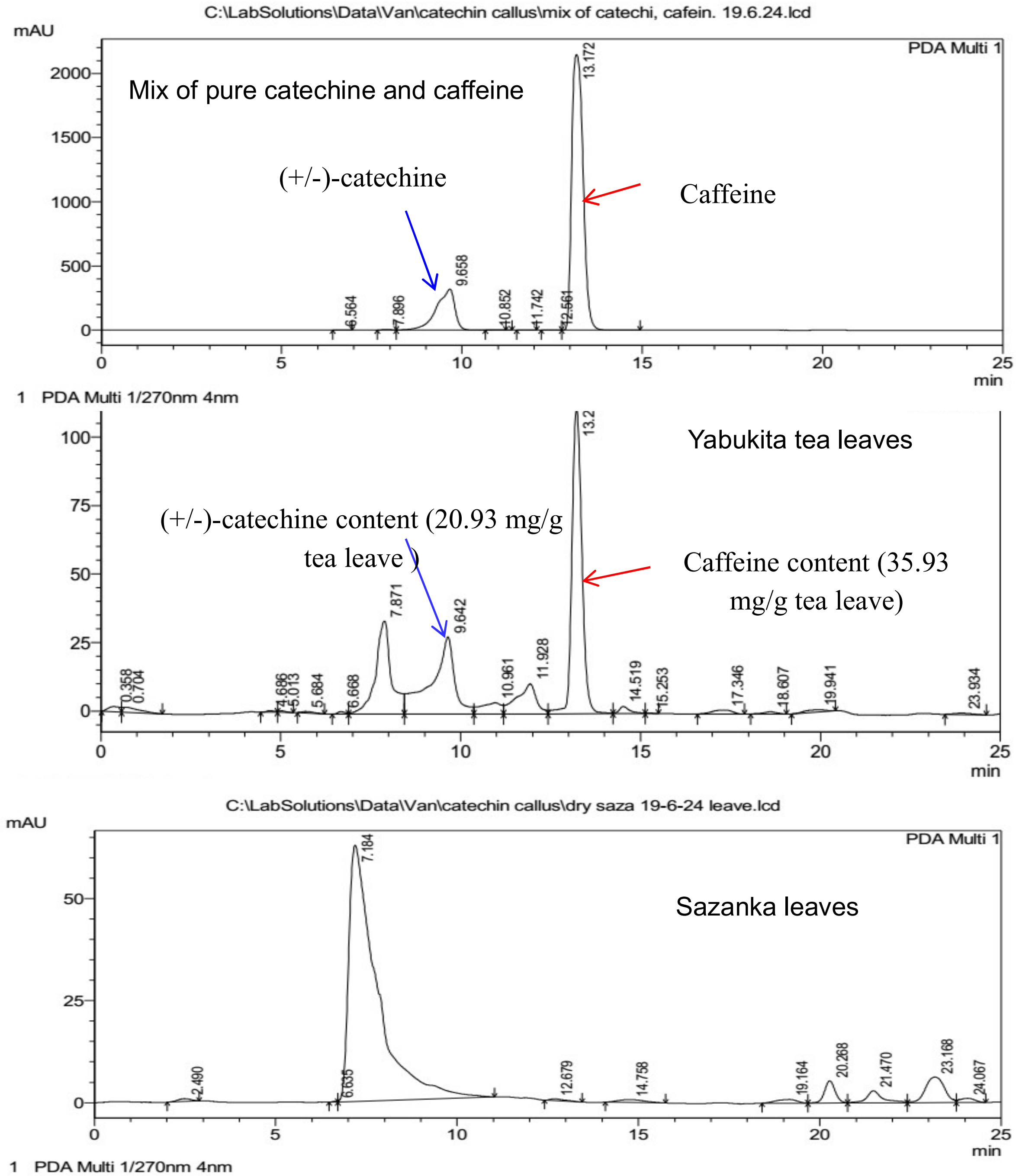


Figure 7.5. Analysis of catechin and caffeine in tea leaves by HPLC

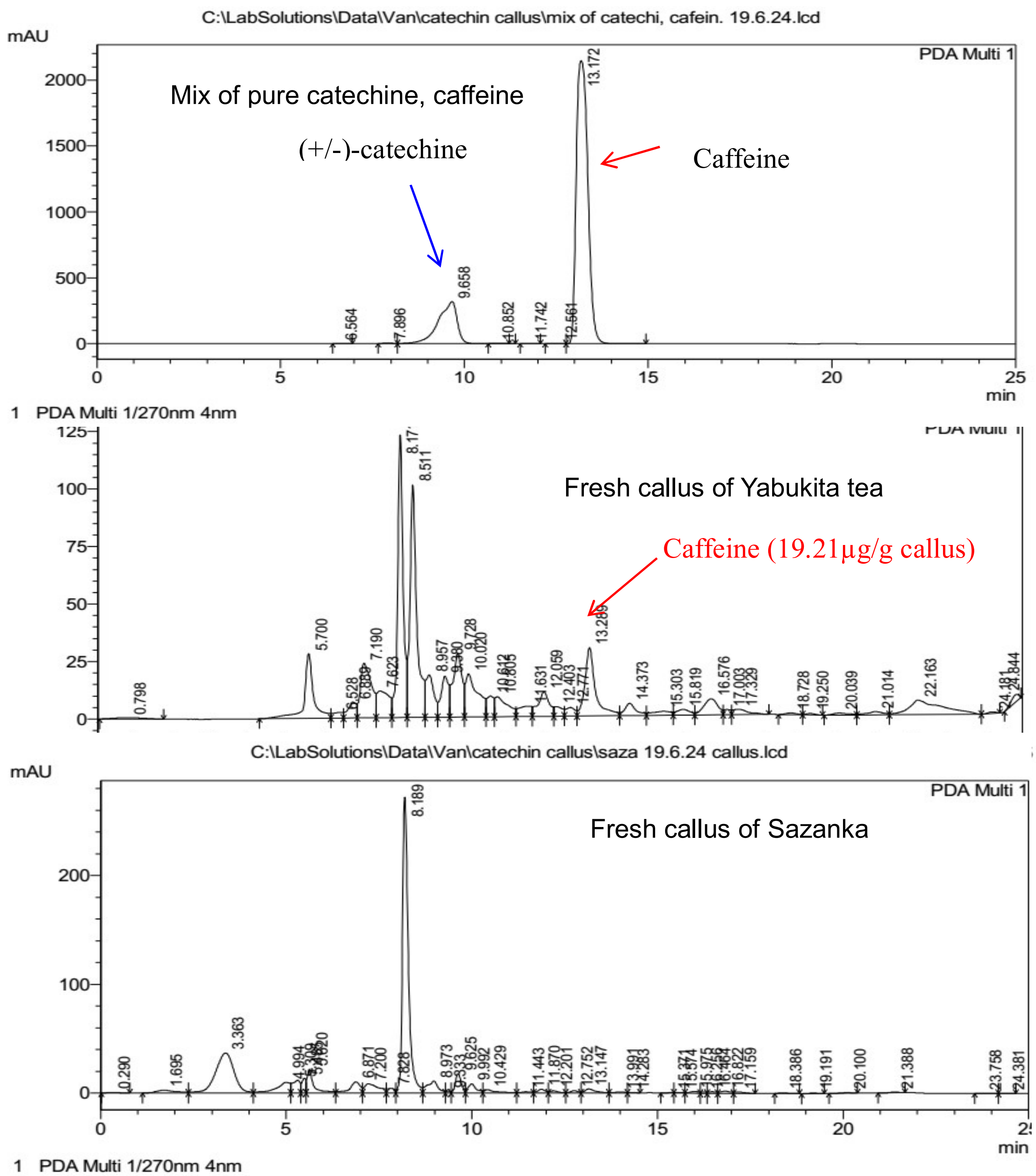


Figure 7.6. Analysis of catechin and caffeine in fresh tea callus by HPLC

CONCLUSION

Results clearly showed that leaching compounds from tea leaves may be considered as potential allelopathic agents. Our results demonstrated the significant differences of allelopathic activity between tea samples, Vinatea- green tea exhibited the greatest inhibitory effect on the growth of lettuce seedling with EC_{50} value at 0.12 mg/ml in sandwich method.

The highest content of caffeine was found in the green tea samples, this content declined in oolong and black tea samples and the lowest content was found in dried tea leaves. Radicle and hypocotyl growth inhibition of lettuce caused by crude extract of tea could be explained by caffeine that estimates to be present in the crude extract of green tea and oolong tea. For dried and black tea, inhibitory effect on the growth of lettuce seedling can relate to other compounds.

Tea extract has been shown to have a strong negative allelopathic potential on germination of some weeds at concentration 75 and 100 mg/ml and the growth of some weeds was strongly inhibited by caffeine selectively. In contrast, caffeine has only weak effect on the growth crops such as rice, barley, carrot.

Caffeine recovery rate in Andosol soil in significantly higher, compared to Fluvisol and Sand soil. The adsorption of caffeine was observed in Fluvisol soil. The caffeine concentration in soil has a positive correlation with radicle growth inhibition of lettuce seedlings. The finding of caffeine concentration at 0.14 µg /g soil proved the presence of caffeine in tea garden, indicated that caffeine may have an ecological significance in plant-plant interaction in soil.

Leaves and callus of Yabukita showed strongly allelopathic effect on the growth of lettuce seedling and caffeine was also considered as a contribution to inhibitory activity of Yabukita tea rather than Tsubaki and Sazanka.

Our results proved that caffeine as allelochemical from tea samples have the potential to suppress the growth of surrounding plants, but its inhibitory properties are variable, depending on types of soils and tested species. It is obvious that caffeine is as a putative allelochemical from tea.

Based on these results, further study about of mode of action caffeine, experiments in greenhouse and field condition can contribute to understand of mechanism of action and practice activity of caffeine allelochemical as natural herbicide.

List of Publication

Pham, V.T.T., Ismail, T., Mishyna, M., Appiah, K.S., Oikawa, Y., Fujii, Y. 2019. Caffeine: The Allelochemical Responsible for the Plant Growth Inhibitory Activity of Vietnamese Tea (*Camellia sinensis* (L.) Kuntze). *Agronomy*, 9, 396

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