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Caffeine: The Allelochemical Responsible for the Plant Growth Inhibitory Activity of Vietnamese Tea (*Camellia sinensis* L. Kuntze)

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Abstract: The present study aimed to examine the phytotoxic potential of seven Vietnamese tea samples based on the specific and total activity of caffeine and tea extracts on test plants. The sandwich method results indicated that the inhibitory effect of tea samples on the radicle and hypocotyl growth of lettuce seedlings was dependent on the concentration and type of tea samples, and also the presence of agar soluble allelochemicals. Among the seven tea samples, the leachates from Vinatea-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. Caffeine concentration in tea samples analyzed by high-performance liquid chromatography (HPLC) varied from 20.7 to 38.2 µg/mL of dry leaves. The specific activity (EC_{50} value) of pure caffeine was 75 µg/mL, and the highest total activity of caffeine estimated in Vinatea-green tea was 0.51 [no unit]. Caffeine from green and oolong tea may be considered as one of the contributors to the inhibitory activity of the crude extract. Moreover, the phytotoxicity of pure caffeine and aqueous tea extracts was highly selective on the growth of different plant species. The concentration of caffeine detected from tea farm soil ranged from 0.137 to 0.145 µg/g soil. The results indicated that caffeine might be considered as a promising allelochemical from Vietnamese tea and can be a good candidate for weed management.

Keywords: allelopathy; caffeine; *Camellia sinensis*; specific activity tea; total activity

1. Introduction

The excessive use of chemical herbicides has caused some environmental pollutions and threatened human health in some extreme situations. Also, the increasing number of herbicide-resistant weeds due to the indiscriminate use of synthetic herbicides could damage the ecosystem over a long period [1–3]. In recent years, allelopathic species and allelochemicals have been utilized as alternative weed management strategies in sustainable agricultural practices [4–6]. 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 [7]. 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 weeds [8,9]. 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 [10].

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that is known for its medicinal properties and also recognized for its essential roles in allelopathic effects against plant species [11] and chemical defense against pathogens and herbivores [12]. 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%) [13]. According to Takeda [14], the concentration of caffeine determined in tea was the highest compared with the other plants such as coffee, cacao, and mate, among others.

A preliminary experiment on tea residue (*Camellia sinensis* L.) extract has previously shown allelopathic potential on some weeds and crop species [15–17]. 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. 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 a 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 [18,19] 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 [20–22]. These findings may serve further study in choosing potential allelochemicals for developing new strategies such as an environmentally friendly herbicide. In the development of new agrochemicals, understanding the species specificity of the identified bioactive compound or the allelochemical is essential for estimating the correct dose of application. However, each kind of weed or crop has different sensitivity to inhibitory compounds from plants [20,23]. Thus, the evaluation of various allelochemicals and using the minimal mixture for weed control purpose may be more effective than the use of a single allelochemical [24].

Vietnam climate and weather conditions are suitable for tea plant growth, and hence, the tea cultivated area and production has increased rapidly over the years. Vuong et al. [25] indicated that Vietnamese green tea had a higher caffeine level than Japanese green tea. With the presence of such significant caffeine content from an abundant tea source, this is an advantage for research for plant allelopathic potential for weed management. Although the residue of tea was reported with allelopathic potential, allelochemicals in the soil can be diminished by absorption by plants, by degradation by photolysis, oxidation, and microbial degradation, and the processes of removal or transfer [26]. Presently, there is no available study of the phytotoxic activity of caffeine from tea in soil concerning its ecological importance. Besides, the detection of allelochemicals in the soil of a growing donor plant is essential for recognizing allelopathy under field conditions, as reported for dehydromatricaria ester (DME) by Ito et al. [27] and Juglone [28].

Furthermore, the concentration of an allelochemical in the soil is a dominant factor directly determining the phytotoxic activity in the soil. In agricultural fields, autotoxicity usually results in a decrease in crop yield and quality. Root exudate from plants is one of the main routes of allelochemical release into the environment, and the exudates can influence the growth of other plants through soils. Ye et al. [29] showed that the soil of consecutively cultivated tea plantations caused significant levels of autotoxicity, and profoundly affected the growth, metabolism, yield, and quality of the transplanted tea seedlings. We hypothesized that the presence of caffeine released from the tea in the soil is a determinant for weed growth inhibition in tea production. In order to understand the role of caffeine under field condition, root exudates and caffeine concentration in the soil extracts were analyzed. In this study, we aimed to (i) evaluate the phytotoxic potentials of Vietnamese tea, (ii) examine the contribution of inhibitory activity of pure caffeine extracted from Vietnamese tea, (iii) test the effect of tea extracts and pure caffeine on the growth of several test plants, and (iv) evaluate the role of caffeine that has been determined in the soil collected from three different locations at a Vinatea-green tea farm.

2. Materials and Methods

2.1. Plant Materials

Fresh tea leaves (V1) (*Camellia sinensis* L. Kuntze) were collected from Thai Nguyen province, Vietnam in December 2014 and then dried at 45 °C for 12 h. Also, six other commercial brands of tea, namely Vinatea-green tea (V2), Vinatea-oolong tea (V3), Thai Binh-LangSon-oolong tea (V4), DucThien-green tea (V5), KimAnh filter bag tea (V6), and Vinatea-black tea (V7) were purchased from the market. 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.

V2: Trung Du variety was collected from Thai Nguyen province, Vietnam from July to August and dried at 100–110 °C for 8 min for the first time and at 80–90 °C for 7 min for the second time.

V3: Kim Tuyen variety was collected from Moc Chau-Son La province, Vietnam from July to August and dried at 90–95 °C for 24 min.

V4: Oolong-ThanhTam variety were collected from Lang Son province, Vietnam from July to August and dried at 90–95 °C for 24 min.

V5: San Tuyet variety was collected from Yen Bai province, Vietnam from July to August and dried at 100–110 °C for 24 min.

V6: Trung Du variety was collected from Ha Giang province, Vietnam from July to August and dried at 90–100 °C for 24 min.

V7: PH1 variety were collected from Phu Tho province, Vietnam from May to October and dried at 105–110 °C for 25 min.

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.), and carrot (*Daucus carota* L.). Monocotyledonous species were: Italian ryegrass (*Lolium multiflorum* Lam.), perennial ryegrass (*Lolium perenne* L.), Timothy (*Phleum pretense* L.), orchardgrass (*Dactylis glomerata* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.) cv. Jhona and Shinshu.

2.2. Screening of Phytotoxic Potential of Tea Samples by the Sandwich Method

The phytotoxic potential of seven tea samples (V1–V7) from Vietnamese tea brands were tested using the sandwich method as described by Fujii et al. [30]. 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. Agar medium without tea samples were used as 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.

2.3. High-Performance Liquid Chromatography (HPLC)

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). 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.

2.4. 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. [22]. The EC_{50} values were determined by a logistic regression equation of the concentration-response curves [31]. 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 min, 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 h, 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.

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

$$\text{Total activity} = \text{caffeine concentration in tea leaves/specific activity of pure caffeine } (EC_{50}). \quad (1)$$

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

2.5. Effect of Aqueous Tea Extracts of Vinatea-Green Tea (V2) on the Germination and Growth of Different Species of Plants

2.5.1. Extraction Procedure

The extraction procedure of tea crude extracts was the same as described in Section 2.4. 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.

2.5.2. Germination Bioassay

One millilitre 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:

$$Germination\ (\%) = (n/t) \times 100 \quad (2)$$

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

2.5.3. 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 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 was extracted with 10 mL distilled water, and after stirring for 10 min, six pure caffeine concentrations were diluted by distilled water into 0, 0.05, 0.1, 0.15, 0.2, and 0.25 mg/mL.

2.5.4. Soil Sampling for Rhizosphere Soil Method

The samples from tea plants (Vinatea-green tea) were taken out from the tea farm. Then, soil adhering to the surroundings of the root "rhizosphere soil" and the soil shaken 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, germination percentage and growth of lettuce seeds were measured [31].

2.5.5. Analysis of Caffeine Residue in the Soil

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. 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.

2.6. 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 and Dunnett multiple comparisons.

3. Results and Discussion

3.1. Screening of Phytotoxic Potential of Tea Samples by the Sandwich Method

The inhibitory effects of leachates from tea leaf samples on the radicle and hypocotyl growth of lettuce seedlings is shown in Table 1. 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. 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. Based on the EC_{50} values, the growth of lettuce seedlings was more highly suppressed by the tea samples from V2 to V6 than tea samples V1 and V7. 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 [32]. 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 [20,33]. 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 [34]. In parallel with Chatterjee et al. [35], 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.

Table 1. The effect of the phytotoxicity of tea samples on the growth of lettuce seedlings by the sandwich method.

			The Concentration of Tea Samples (mg of dried leaves/ mL of agar)						
Samples	Tea type		0.05	0.1	0.2	0.5	1	5	
			Percentage of radicle growth of lettuce seedling compared to control						*EC50
V1	Fresh tea	R	98.21 ± 13.78	94.64 ± 10.5	71.55 ± 14.78	41.42 ± 7.95	28.02 ± 8.46	10.15 ± 4.36	0.40a
		H	103.41± 1 2.23	110 ± 17.82	101.02 ± 15.7	103.67 ± 12.6	88.23 ± 16.81	52.94 ± 14.03	5.10a
V2	Green tea	R	87.66 ± 10.21	52.19 ± 17.56	35.14 ± 14.96	22.94 ± 8.90	17.05 ± 10.61	4.46 ± 1.55	0.12c
		H	100.11 ± 8.46	92.27 ± 18.55	76.32 ± 17.12	66.17 ± 25.46	54.26 ± 25.4	23.38 ± 9.42	1.00c
V3	Oolong tea	R	90.42 ± 9.84	76.37 ± 10.03	40.86 ± 8.44	34.92 ± 10.02	18.27 ± 4.83	5.07 ± 2.18	0.23c
		H	99.28 ± 13.53	93.63 ± 19.70	94.85 ± 13.15	92.20 ± 18.29	65.29 ± 23.43	18.97 ± 12.17	2.20bc
V4	Oolong tea	R	85.47 ± 11.41	53.09 ± 16.23	35.94 ± 9.22	31.47 ± 7.69	20.50 ± 5.63	5.27 ± 1.37	0.17c
		H	98.46 ± 14.1	101.81 ± 14.45	95.73 ± 21.78	87.35 ± 18.75	67.94 ± 15.82	13.67 ± 7.47	1.40bc
V5	Green tea	R	89.75 ± 7.23	56.36 ± 12.78	31.37 ± 8.25	20.50 ± 5.57	21.31 ± 8.78	3.65 ± 1.24	0.16c
		H	100.57 ± 12.06	99.54 ± 14.06	90.88 ± 17.05	63.97 ± 21.46	65.73 ± 23.12	12.79 ± 3.50	2.10bc
V6	Green tea	R	88.65 ± 8.24	68.46 ± 11.13	35.98 ± 18.02	22.94 ± 5.38	17.055 ± 3.38	3.45 ± 1.06	0.21c
		H	104.66 ± 10.26	92.72 ± 15.62	84.26 ± 21.44	84.70 ± 12.80	51.61 ± 13.63	11.91 ± 2.81	2.20b
V7	Black tea	R	90.55 ± 13.27	80.44 ± 14.18	72.28 ± 12.58	57.46 ± 13.17	59.69 ± 16.14	13.40 ± 3.74	1.90b
		H	106.24 ± 15.72	112.05 ± 20.66	100.58 ± 17.41	113.82 ± 16.24	107.72 ± 20.08	50.05 ± 14.65	5.00a

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.

3.2. Determination of Caffeine Concentration in Tea Samples

The concentration of caffeine was found to be in the range of 20.7 to 38.2 µg/mL among the tea samples by HPLC analysis (Table 2). In the group of green tea samples, caffeine concentration ranged from 26.0–38.2 µg/mL; in oolong tea 21.4–23.3 µg/mL, 26.1 µg/mL in black tea, and 20.7 µg/mL in fresh tea. 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 [36]. 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. [37] in which the caffeine concentration in fresh tea leaves ranged from 2.06% to 4.68% in dry matter. According to Ashihara and Kubota [38], 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 [39].

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 [40] 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 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.

3.3. Specific and Total Inhibitory Activity

3.3.1. 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 1 shows the inhibitory effect of pure caffeine (at the same concentration obtained in the crude extract) and crude extracts of V1, V2, V4, and V7 on the radicle growth of lettuce seedlings (V3, V5, and V6 were almost similar to V4 in inhibitory effect). 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 in order to find the contribution of the caffeine into the total inhibitory activity of crude tea extract, and then choose the best treatment for tea allelopathy. The pure caffeine inhibitory effect on lettuce radicle growth was calculated by plotting the pure caffeine concentrations and its corresponding radicle growth. Based on concentration of caffeine of each tea sample in 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 1 reflects that inhibitory activity curve of V2, V4, and pure caffeine has similar shapes of curves than that of V1 and V7. Thus, caffeine accounts for most of the inhibitory activity caused by the crude tea extract of V2 and V4, 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 crude tea extract, caffeine EC_{50} values were 2.44 and 3.48 mg/mL in V2

and V4, respectively. Almost all the values considered are closer to the EC_{50} value of the crude tea extract than that of V1 and V7. From this information, caffeine may be the main contributor to the inhibitory activity of V2 and V4. Similarly, the result of Takemura et al. [41] 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 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 Vinatea-oolong tea samples.

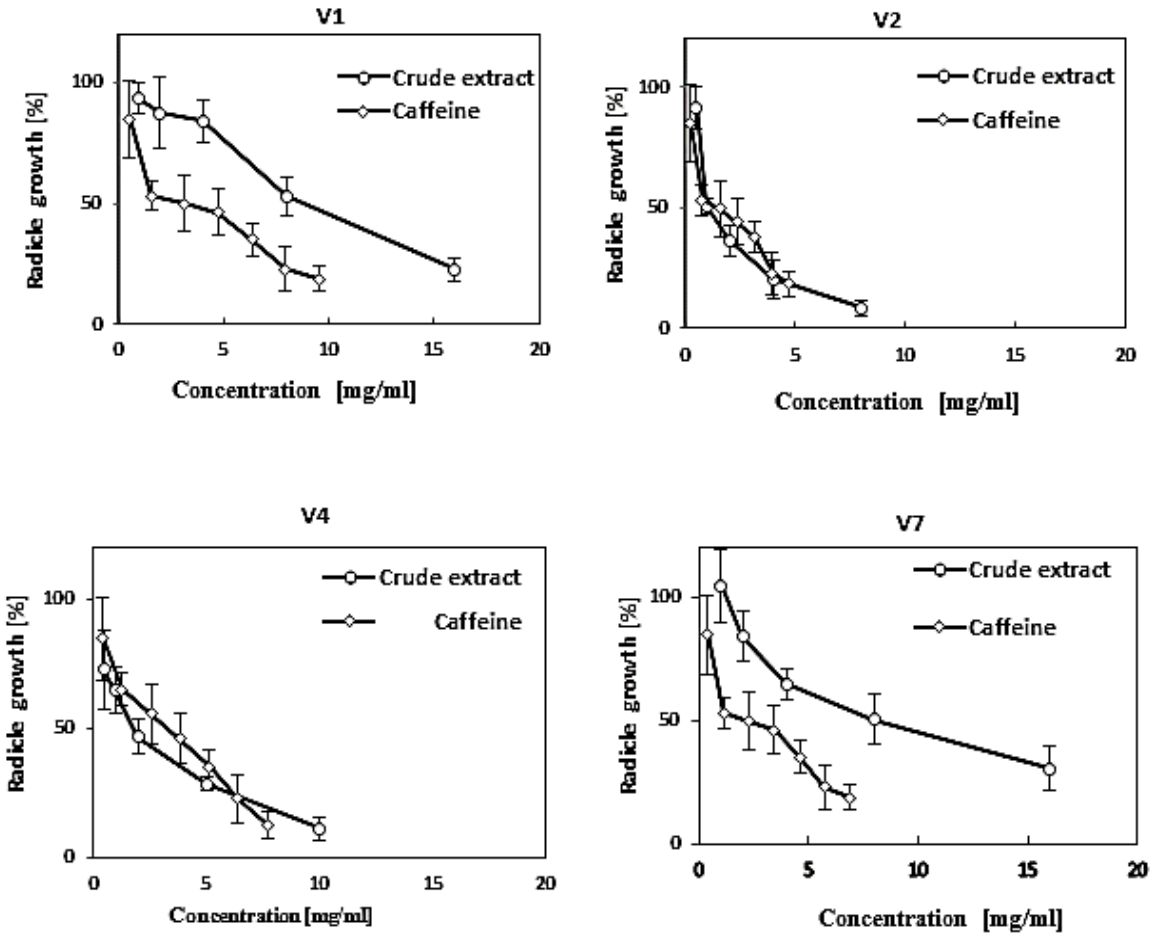

Figure 1. Inhibition activity of the radicle growth of lettuce seedlings caused by the crude extract of tea and by caffeine estimated to be present in crude tea extract. The data are the mean of three replications ± SD.

3.3.2. 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 [18]. Based on the result of HPLC analysis, from Equation (1) the total activity of caffeine in V2 sample = 38.2 µg/mL/75 µg/mL was 0.51, resulting in the highest activity compared to the other tea samples (0.27 to 0.51) due to its greater total activity value (Table 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) [19]. Therefore, Vinatea-green tea (V2) can be considered as a potential material with the inhibitory substance being caffeine.

Table 2. 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 (µg/mL) (*Camellia sinensis*)	Total Activity (no unit)
V1	Fresh tea	10.2 ^{a}	20.7 ^{f} (±0.02)	0.27
V2	Green tea	1.22 ^{b}	38.2 ^{a} (±0.06)	0.51
V3	Oolong tea	1.31 ^{b}	21.4 ^{e} (±0.06)	0.29
V4	Oolong tea	1.98 ^{b}	23.3 ^{d} (±0.24)	0.31
V5	Green tea	1.56 ^{b}	35.5 ^{b} (±0.23)	0.47
V6	Green tea	2.10 ^{b}	26.0 ^{c} (±0.15)	0.35
V7	Black tea	10.2 ^{a}	26.1 ^{c} (±0.02)	0.35

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.

3.4. 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 all the seed germination of crops and weed at 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 result showed that the germination percentage of the test species depended on the concentration of tea extract. The effect on germination percentage by phytotoxic plants extracts in a dose-dependent manner was also reported in previous studies [42,43]. Nonetheless, Jhona rice cultivars were found to be highly resistant to tea extract at all tested concentrations, and the germination percentage was affected at very high concentration at 100 mg/mL (Figure 2). 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 [11].

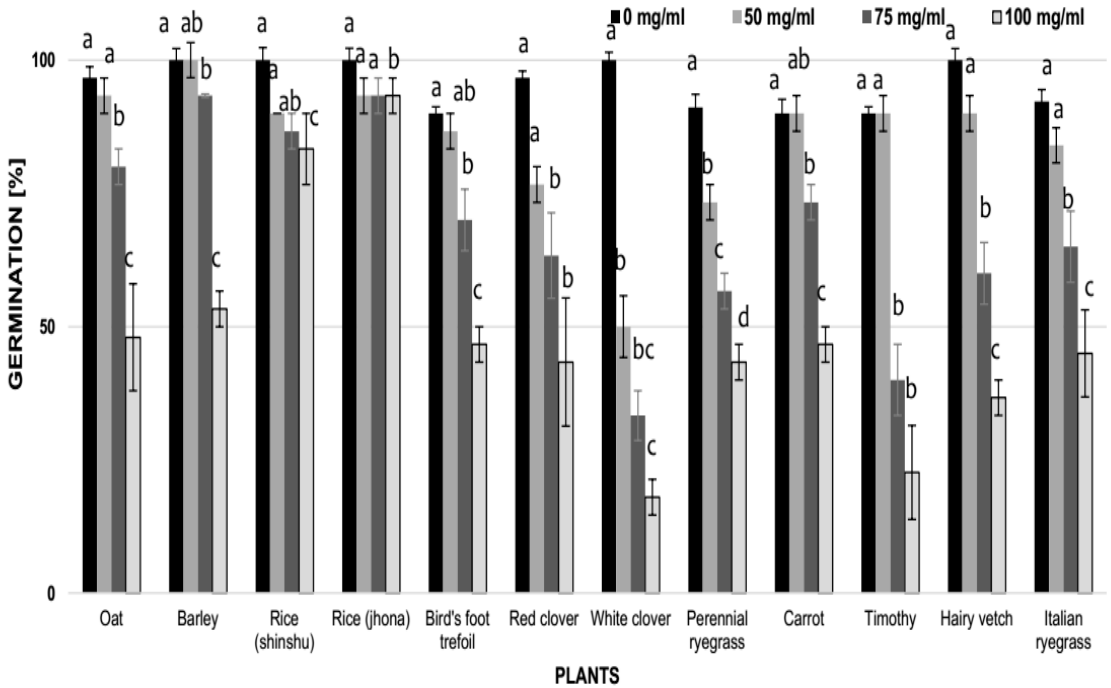

Figure 2. The effect of Vinatea-green tea extract on the germination of crops and weeds. Columns followed by the same letter, within the same crop or weed, are not significantly different ($p < 0.05$, Tukey and Dunnett test). The data are the mean of three replications ± SD.

The effect of Vinatea-green tea extract and pure caffeine on the radicle growth of crops and weeds is summarized in Table 3. 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 birdsfoot 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. Khursheed et al. [44] 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.) [20]. From the present preliminary investigation, it can be concluded that green tea leaves exhibited remarkable phytotoxic potential by significantly affecting 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 [45]. 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 [46]. One of the suggested reasons is that inhibition of germination by caffeine causes a marked decrease in amylase activity [11]. 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 [15,17].

Table 3. Effect of Vinatea-green tea extraction and caffeine on radicle growth of the crop and selected weed species.

Scientific Name (English Name) (family [a])	EC_{50} (mg/mL) [b]	
	Crude Extract	Pure Caffeine
Phleum pratensis (Timothy) (po)	1.24 ^{a}	0.15 ^{ab}
Trifolium repens (White clover) (fa)	1.12 ^{a}	0.10 ^{a}
Trifolium pretense (Red clover) (fa)	3.01 ^{ab}	0.10 ^{a}
Hordeum vulgare (Barley) (po)	4.34 ^{bc}	0.15 ^{ab}
Lotus corniculatus (Birdsfoot trefoil) (fa)	5.06 ^{c}	0.10 ^{a}
Lolium perenne (Perennial ryegrass) (po)	5.62 ^{c}	2.50 ^{b}
Lolium multiflorum (Italian ryegrass) (po)	5.17 ^{c}	2.50 ^{b}
Dactylis glomerata (Orchard grass) (po)	5.91 ^{c}	2.50 ^{b}
Avena sativa (Oat) (po)	6.02 ^{c}	2.50 ^{b}
Vicia villosa (Hairy vetch) (fa)	8.32 ^{d}	>2.50 ^{c}
Daucus carota (Carrot) (ap)	8.64 ^{de}	>2.50 ^{c}
Oryza sativa, cv. Jhona (Rice) (po)	8.82 ^{de}	>2.50 ^{c}
Oryza sativa, cv. shinshu (Rice) (po)	10.4 ^{e}	>2.50 ^{c}

[a] Abbreviations of family names are as follows: po = Poaceae, fa = Fabaceae, ap = Apiaceae; [b] 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). The data are the mean of three replications ± SD.

3.5. 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 (Figure 3). Thus, compounds released by Vinatea-green tea plant roots and adsorbed into rhizosphere soil particles showed phytotoxic potential.

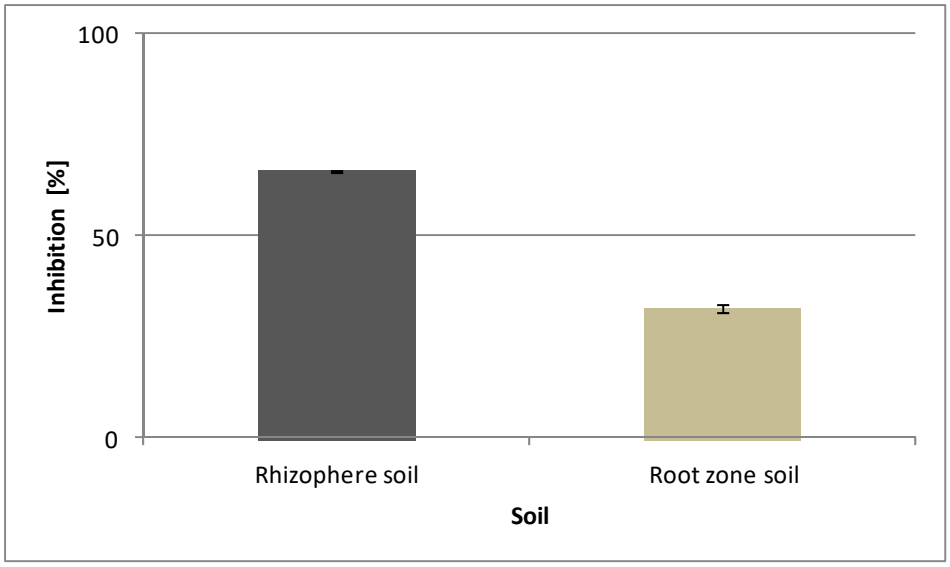

Figure 3. The effect of the phytotoxicity of rhizosphere soil and root-zone soil on the growth of lettuce seedlings by the rhizosphere soil method. The data are the mean of three replications ± SD.

Also, to confirm the existence and availability of caffeine in the soil of V2, the concentration of caffeine have been measured by HPLC. The analytical results showed that caffeine concentration ranged from 0.137 to 0.145 μg/g soil (Table 4), and recovery and reproducibility for caffeine extraction from spiked soil were satisfactory (approximately 75%). 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 [27], 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 its concentration in soil water.

Table 4. The concentration of caffeine from the soil collected from three different locations at Vinatea-green tea farm.

Soil Samples	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 ± SD.

4. Conclusions

All the Vietnamese tea samples tested showed plant growth inhibitory potential by affecting the radicle growth of lettuce seedlings. The present study revealed that caffeine could be partly responsible

for the phytotoxic effect of tea extracts, and Vinatea-green tea may be considered as a potential material for future weed management. Our findings revealed the selective weed inhibitory properties of aqueous extraction of Vinatea-green and caffeine, suggesting it could be agriculturally significant for sustainable weed management. This study has demonstrated the presence of caffeine in the soil of the tea garden. However, the contribution in recognizing allelopathy as well as the mechanism of caffeine activity under field condition needs to do in the next steps.

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