Chapter 6

Influence of Exogenously Supplemented Caffeine on Cell Division, Germination, and Growth of Economically Important Plants

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Abstract

Caffeine is a plant secondary metabolite of antiherbivory, allelopathic, and antibacterial activity. In our previous study, caffeine was shown to be an effective agent toward plant pathogenic bacteria causing high economic losses in crop production worldwide. Current study indicated that growth media supplementation with soil or plant extract did not interfere with antibacterial action of caffeine against *Clavibacter michiganensis, Dickeya solani, Pectobacterium atrosepticum, Pectobacterium carotovorum, Pseudomonas syringae, Ralstonia solanacearum, and Xanthomonas campestris*. The impact of caffeine on plant cell division, seed germination and growth of economically important plants was evaluated to assess possible applicability of caffeine in plant protection field. Caffeine impaired plant cell division process and inhibited *in vitro* germination of tomato and lettuce. Regeneration of potato explants was also negatively affected by the addition of caffeine. However, caffeine spraying or watering of tomato, lettuce and cabbage grown in soil did not impair plant development. Although the tested plants accumulated caffeine, its inner quantity was reduced by peeling and/or cooking. According to the results, caffeine warrants additional attention as a useful, natural compound designated for the control of bacterial plant pathogens. Proposed treatment seems promising especially in the case of providing protection for overwinter-stored table potato tubers.

Keywords: antimicrobials, *Brassica oleracea*, *Clavibacter* sp., *Dickeya* sp., *Lactuca sativa*, *Pectobacterium* sp., plant protection, *Pseudomonas* sp., *Ralstonia* sp., *Solanum lycopersicum*, *Solanum tuberosum*, *Vicia faba*, *Xanthomonas* sp.

1. Introduction

Plants produce a broad range of secondary metabolites exhibiting antibiotic, antifungal, antiviral, antigerminative, allelopathic, UV light absorbing, insecticidal, or even antiherbivore activities [1]. Caffeine (1,3,7–trimethylxanthine) is one of over 12,000 alkaloids of plant origin [2]. So far, caffeine has captured attention for its pharmacological activity, being the most widely consumed psychoactive substance in the world [3]. But little is known about its potential application in plant protection. Until now, it was reported that caffeine could be used to eradicate or repel molluscs, insects, frogs, or birds [4–7]. Also, the antibacterial activity of caffeine toward microbes inhabiting different ecological niches was demonstrated. This substance impaired growth of human pathogens like *Escherichia coli* O157:H7 responsible for approximately 73,500 cases of foodborne illnesses per year [8], constituents of natural human microflora such as *Streptococcus mutans* [9], or terrestrial and aquatic inhabitants like *Pseudomonas fluorescens* [10]. To the best of our knowledge, caffeine bactericidal properties against plant pathogenic bacteria have been examined so far by a few groups only. Kim and Sano [11] inoculated transgenic tobacco plants producing 1.8 μg caffeine per gram of fresh weight with *Pseudomonas syringae* pv. *glycinea* and noted remarkably lower disease severity in comparison with the nontransgenic plants. As many problems arise with the approval of genetically modified organisms, scientific attention focused on exogenously applied caffeine. Caffeine direct bactericidal action against *P. syringae* pv. *glycinea* was correlated with the increasing concentration of this compound [11]. Subsequently, Sledz et al. [12] evaluated antibacterial activity of caffeine toward broad spectrum of plant pathogenic bacteria causing economic losses in crop and ornamental plant production worldwide: *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), *Dickeya solani* (Dsol), *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pseudomonas syringae* pv. *tomato* (Pst), *Ralstonia solanacearum* (Rsol), and *Xanthomonas campestris* pv. *campestris* (Xcc). Caffeine inhibited growth of the above-listed phytopathogens in broth cultures, increased their generation time, triggered morphological abbreviations, and finally exhibited bactericidal effect in concentrations from 40 to 100 mM [12]. Moreover, application of this compound reduced disease symptoms caused by Dsol on potato slices, whole potato tubers, and chicory leaves [12]. In addition, it was shown that the plant pathogenic bacterium tested could not develop any resistance to the caffeine treatment [12].

Taking into account these data, caffeine seems to be a promising antimicrobial agent that might be utilized in the plant protection field, especially because of the limited amount of possible alternatives [13]. In the past, worldwide spread of multidrug-resistant microorganisms suggested more prudent uses of antibiotics in agriculture [14], thus possible plant control approaches seem even more restrained nowadays. In general, mostly preventive procedures are implemented to reduce economic damage triggered by plant pathogenic bacteria in the field, transportation, or storage [13].

In this work, we undertook further studies on evaluating possible applicability of caffeine as an antimicrobial agent to be used in agriculture. We investigated whether caffeine retains its action

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against plant pathogenic bacteria Cms, Dsol, Pba, Pcc, Pst, Rsol, and Xcc in the presence of substances occurring in soil or plant extracts. Moreover, the impact of caffeine treatment on plants of economic importance was studied. The effect of caffeine supplementation on plant cell division was shown in the sprouts of broad bean. Furthermore, we evaluated the influence of caffeine on plant germination and growth both *in vitro* and in soil. Last but not least, caffeine accumulation in the tested plants was investigated. In addition, the effect of peeling and/or cooking on inner caffeine content in potato tubers was evaluated. Altogether, this study provides an insight into possible ways of exploiting antibacterial activity of caffeine in plant protection field.

2. Materials and methods

2.1. Bacterial strains

Strains of plant pathogenic bacteria used in this study are: *Clavibacter michiganensis* subsp. *sepedonicus* LMG 2889, *Dickeya solani* IFB 0099, *Pectobacterium atrosepticum* SCRI 1043, *Pectobacterium carotovorum* subsp. *carotovorum* SCRI 180, *Pseudomonas syringae* pv. *tomato* LMG 5093, *Ralstonia solanacearum* LMG 2294, and *Xanthomonas campestris* pv. *campestris* LMG 582.

2.2. Plant material

The following plants were used: lettuce (*Lactuca sativa* L. var. *capitata*, cv. Queen of May), tomato (*Solanum lycopersicum* L., cv. Baron, cv. Betalux), cabbage (*Brassica oleracea* L. *convar. capitata*, cv. First harvest), potato (*Solanum tuberosum* L., cv. Irga, or the breeding lines: LB-6 and LB-12 [15]), and broad bean (*Vicia faba* L., cv. Hangdown white).

2.3. Growth media and media with soil or plant extract supplementation

To prepare soil extract, 1000 g of Substral soil (Scotts, Warsaw, Poland) was mixed (30 min, 250 rpm) with 2000 ml of distilled water. The suspension was filtered through Whatman paper grade 1 (Sigma-Aldrich, St. Louis, USA), and the resulting filtrate was autoclaved for 30 min.

On the basis of soil extract, soil medium was prepared as follows: 200 ml of the soil extract was supplemented with 1 g of K_3PO_4, 2 g of NaCl, 0.5 g of NH_4NO_3, and 1 g of glucose. pH was adjusted to 7.2.

Potato, tomato, and cabbage extract media were prepared as listed here: 50 g of potato tubers, 7.5 g of tomato leaves, or 20 g of cabbage leaves were homogenized in 100 (potato) or 20 ml (cabbage and tomato) of Ringers buffer in extraction bags (Bioreba, Basel, Switzerland). In the case of potato tissue extract 0.02 g of diethyldithiocarbamic acid was added. Then the homogenates were centrifuged at 4000 rpm for 15 min. The supernatant was collected and supplemented with additional nutrients to culture Pst (0.1 g of glucose) or Rsol (0.1 g of glucose and 0.3 g of yeast extract). Plant extract media were sterilized in sequence with 5, 1.2, 0.8, 0.45, and 0.22 μm Minisart SRP Syringe Filters (Sartorius, Goettingen, Germany).

In vitro plant cultures were conducted on MS [16], ½ MS or basic plant growth medium (sucrose 30 g l^{-1} and agar 7.5 g l^{-1}) depending on the experiment.

2.4. Effect of soil and plant extracts on the antibacterial action of caffeine

To examine whether substances present in soil or plant extracts could impede antibacterial activity of caffeine toward plant pathogens, the growth of Cms, Dsol, Pba, Pcc, Pst, Rsol, and Xcc cultures in soil extract medium and plant extract medium containing 0, 5, 10, or 0, 1, 3 mM caffeine, respectively, was monitored for over 24 h by measuring the relative change in OD_{580}. Choice of the plant extract medium for testing the survival of a specific pathogen was based on the preferable host. Potato extract medium was used in the case of Cms, Dsol, Pba, Pcc, and Rsol. Pst was cultured in tomato extract medium, while Xcc was incubated in cabbage extract medium. The experiment was conducted in darkness at 28°C (the exception was Cms cultured at 21°C).

2.5. Effect of caffeine on plant cell division

Broad bean seeds were incubated in distilled water for 24 h (20 seeds per 200 ml) and then germinated on moistened Whatman paper at 20°C. The sprouts were transferred to Petri plates containing six layers of lignin and watered with 0 or 8 mM caffeine. The sprouts were then kept at 24°C for 72 h. After the sprouts were washed, their apical meristems were isolated and cut into 5-mm slices, which were fixed and stained according to the Feulgen protocol [17]. There were four control and four treated samples and one to two preparations per sample. Cells were observed with a light microscope at 500 to 1600× magnification. For each preparation, 1000 cells were selected at random and examined for exhibited mitotic phase and visible micronuclei as described by Evans et al. [18]. The following parameters were determined: the mean mitotic index (the percentage of dividing cells in the observed cell population), phase index (the percentage of cells in prophase, metaphase, anaphase, or telophase), and the frequency of micronuclei. The experiment was performed twice.

2.6. Effect of caffeine on seed germination

The seeds of lettuce (cv. Queen of May) and of tomato (cv. Betalux) were surface-sterilized in 5% $Ca(OCl)_2$, rinsed with sterile-distilled water, and then placed in Petri dishes (10 seeds per dish) on Whatman filter papers moistened with 5 ml of a caffeine solution at 0, 1, 3, 5, 8, 10, 15, or 20 mM. Each combination of seed type and caffeine concentration was represented by at least five Petri dishes, which were sealed with parafilm and kept at 24°C with a 16/8 h light/dark photoperiod. Germinated and non-germinated seeds were counted after 3 or 7 days in the case of lettuce and tomato, respectively. The experiment was performed twice.

2.7. Impact of caffeine on early growth of seedlings

The effect of caffeine on *in vitro* germination of cabbage (cv. First harvest) and tomato (cv. Baron and cv. Betalux) seeds and on the early growth of seedlings was evaluated by placing surface-sterilized seeds on basic plant growth medium supplemented with 0, 1, 5, or 8 mM caffeine.

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After 10 days at 24°C with a 16/8 h light/dark photoperiod, germinated seedlings were transferred to ½ MS medium with the same caffeine concentrations as before. Plant growth and development were monitored for 1 month. Each combination of cabbage or tomato seeds and certain caffeine concentration treatment was represented by four plants, and the experiment was performed three times.

2.8. Effect of caffeine on explants regeneration

Explants of potato (LB-6 and LB-12) stem fragments were transferred to MS medium containing 0, 1, 5, or 8 mM caffeine, and their growth was monitored for 6 weeks at 24°C (16/8h light/dark photoperiod). After the experiment, plant heights were measured. Each combination of potato line and caffeine concentration was represented by three replicates. The experiment was performed three times.

2.9. Effect of caffeine spraying and watering on soil-grown plants

The spraying experiment included cabbage (cv. First harvest), lettuce (cv. Queen of May), and tomato (cv. Betalux). The seeds were germinated on moistened Whatman paper, and after 2 weeks the seedlings were planted in pots (27 × 31 × 4 cm) containing autoclaved soil. There were five rows of 10 plants per pot. The pots were kept at 20°C with a 16/8 h light/dark photoperiod and were watered every 3 days. After the seedlings had been grown in the pots for 10 days, they were sprayed (10 ml per pot) with an aqueous solution containing 0, 1, 5, or 8 mM caffeine. The caffeine was applied seven times over 6 weeks before plant heights were measured. The experiment was performed three times.

The effect of watering with caffeine was assessed on tomato (cv. Betalux) and lettuce (cv. Queen of May). Seeds were planted in pots (7 × 7 × 12 cm; nine seeds per pot and four pots per plant type) containing autoclaved soil. The plants were kept at 20°C with a 16/8 h light/dark photoperiod. Each pot was watered every 3 days with 30 ml of caffeine solution (0, 1, 5, or 8 mM). After 4 weeks, plant heights were measured. The experiment was performed twice.

2.10. Caffeine accumulation in plant tissue

Plants collected from experiments concerning the effects of caffeine on plant germination, growth, and development were examined for caffeine accumulation. Plant material was frozen and stored at −20°C. Later on, it was gently thawed, washed twice with distilled water, dried, and weighed. Afterwards, the tissue was frozen in liquid nitrogen and crushed into small pieces. Two extracts per sample were prepared in 1 ml of Milli-Q H_2O by heating two-thirds of the sample to 100°C for 20 min. A third extract was obtained by keeping one-third sample at 25°C for 20 min. All three extracts were pooled and filtered via a 0.45-μm Minisart SRP Syringe Filter (Sartorius). The filtrates were kept at 4°C before they were processed by high performance liquid chromatography (HPLC) with a Series 200 system (Perkin Elmer, Waltham, USA) and a C18 column (Sigma-Aldrich). A 15% methanol solution was used as a mobile phase. The retention time of caffeine was about 8.3 min.

Caffeine content in the samples was determined by calculating the surface area under the 280 nm absorbance peak in comparison to a standard curve obtained with different caffeine concentrations.

2.11. Impact of peeling and/or cooking on caffeine content

Five potato tubers (cv. Irga) were incubated in a 100 mM caffeine solution for 24 h at room temperature. Samples (0.8 g each) were collected from the peels and from the transitional and middle zones. Potato middle zone was a cube of approximately 3 × 3 × 3 cm originating from the center of the inner mass. Transitional zone enclosed between the middle zone and the peel. Caffeine was extracted from the potato tuber samples with dichloromethane. Caffeine content was assessed in the zones by gas chromatography (Clarus 600, Perkin Elmer) and the quantity of caffeine per gram of dry weight was subsequently calculated.

Other five potato tubers were incubated at room temperature in a 100 mM caffeine solution for 24 h and then cooked, with or without the peels, at 100°C for 20 min. Samples were collected and the caffeine content in specific zones was evaluated as described above.

2.12. Statistical analysis

Statistical significance within plant germination experiments was evaluated by Kruskal-Wallis test, followed by Dunn's test, while the impact of caffeine spraying and watering on plant heights was assessed with the Tukey's test (HSD). $p < 0.05$ was utilized.

3. Results and discussion

3.1. Effect of soil and plant extract on the antibacterial activity of caffeine

The growth dynamics of Dsol, Cms, Pba, Pcc, Pst, Rsol, and Xcc in soil extract media and plant extract media supplemented with 0, 5, 10 and 0, 1, 3 mM caffeine, respectively, was evaluated. Caffeine still reduced bacterial growth in such conditions (**Figure 1**). Interestingly, caffeine was the most effective against Xcc, Rsol, and Cms both in the case of soil and plant extract supplemented media (**Figure 1**). Observed inhibition pattern for all the tested pathogens was similar to the one reported by Sledz et al. [12]. It needs to be taken into account that the examined plant or soil extracts were autoclaved prior to use, and the metabolic activity of soil and plant microflora has also an impact on vastness and diversity of substances naturally occurring in the environment. Further research is needed to exclude possible inactivation of caffeine via complex formation with polyphenols or sequestration into chlorogenic acid complex [19]. Likewise, the impact of species capable of caffeine degradation, e.g. *Pseudomonas cepacia, Pseudomonas putida*, and *Serratia marcescens*, needs to be taken into consideration. In addition, the metabolites present in the

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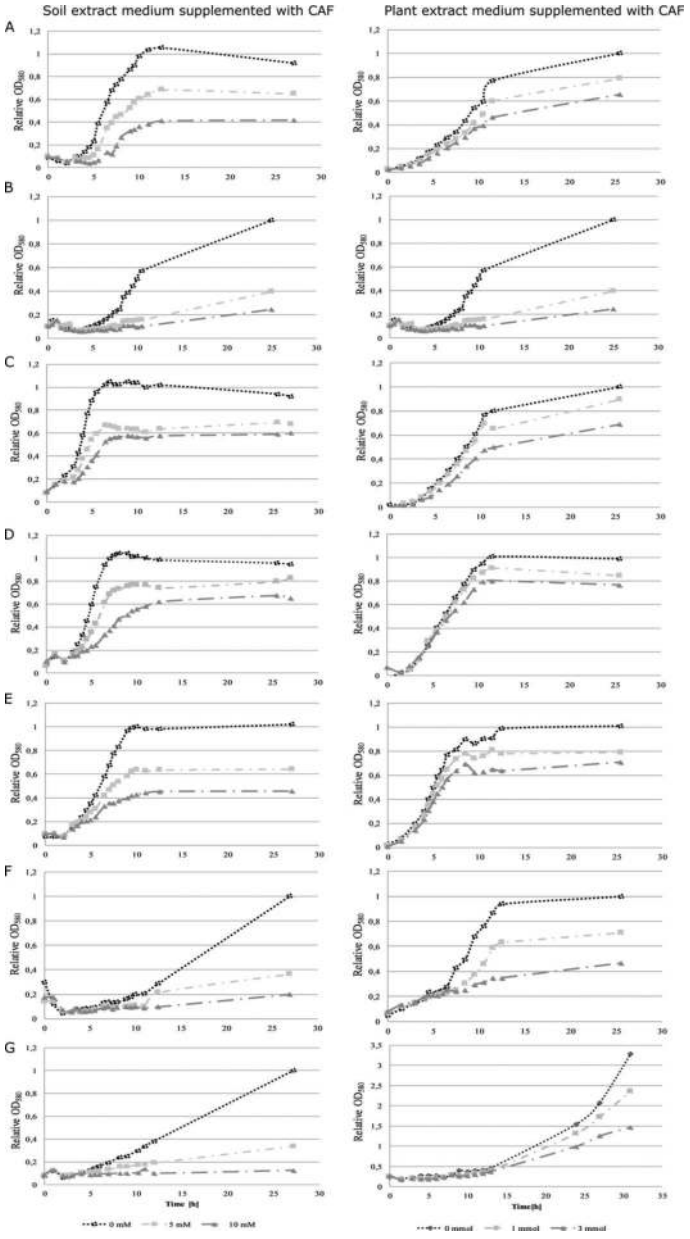
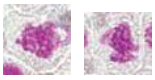
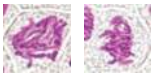
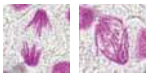


Figure 1. Influence of soil and plant extract on the antibacterial activity of caffeine against the following phytopathogens: (A) *Dickeya solani*, (B) *Ralstonia solanacearum*, (C) *Pectobacterium atrosepticum*, (D) *Pectobacterium carotovorum* subsp. *carotovorum*, (E) *Pseudomonas syringae* pv. *tomato*, (F) *Xanthomonas campestris* pv. *campestris*, and (G) *Clavibacter michiganensis* subsp. *sepedonicus*.

implemented plant and soil extracts are in more oxidized state than those enclosed inside plant tissue.

3.2. Influence of caffeine on plant cell division

To evaluate the effect of exogenous caffeine supplementation on plant cell division, we used *Vicia faba* L. model, widely utilized in studies on environmental mutagens [20]. The mitotic index in broad bean apical meristems was increased by 8 mM caffeine treatment in comparison with the non-treated controls (**Table 1**). This resulted mainly from higher percentage of cells undergoing the prophase state. Lack of caffeine treatment resulted in higher percentage of cells in later stages of cell division process, namely, metaphase, anaphase, and telophase. Besides, micronuclei were observed more frequently in the caffeine-treated cells than in the non-treated samples. Altogether, our results indicate that caffeine treatment resulted in higher frequency of cells undergoing earlier phases of cell division and having visible micronuclei, which points into symptoms of genome instability. Premature chromosome condensation resulting in apoptosis-like programmed cell death was postulated by Rybaczek et al., while investigating caffeine action on root meristems of *Vicia faba* [21]. Interestingly, Friedman and Waller [22] reported repression of mitosis and cell plate formation in coffee seeds exposed to 10 mM caffeine, while Valster and Hepler [23] observed that caffeine allows initiation of the cell plate formation but prevents its completion in living *Tradescantia* stamen hair cells. According to Valster and Hepler, the cytokinesis is affected by the inhibition of cytoskeletal torus formation during phragmoplast expansion [23].

Cell division parameter	Caffeine concentration	
	0 mM	8 mM
Mitotic index (%)	7.27 ± 0.5	9.53 ± 0.34
Prophase index (%) 	43.83 ± 8.67	68.57 ± 2.76
Metaphase index (%) 	35.19 ± 6.46	26.69 ± 5.98
Anaphase index (%) 	11.40 ± 0.47	2.97 ± 3.43
Telophase index (%) 	9.57 ± 1.91	1.77 ± 2.28
Micronuclei frequency (%) 	0.39 ± 0.12	2.71 ± 1.18

~3800 and ~1600 cells were analyzed for 0 and 8 mM caffeine treatment, respectively. Presented values are means ± SD.

Table 1. Influence of exogenous caffeine application on broad bean cell divisions.

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3.3. Effect of caffeine on seed germination and plant development

In order to evaluate possible ways of applying caffeine against bacterial phytopathogens, the effect of caffeine on seed germination and early plant development *in vitro* was assessed. Caffeine reduced the germination rate of lettuce and tomato seeds on caffeine-moistened Whatman paper in a dose-dependent manner (**Figure 2**). Application of caffeine in concentrations higher than 5 and 8 mM significantly reduced the germination rate of lettuce and tomato, respectively, in comparison with the non-treated controls. This observation corresponds with studies on coffee seeds, as caffeine released from fallen, decomposing leaves of mature trees was proven to inhibit seed germination in the coffee plantations [22]. On the contrary, Avery et al. [7] found that caffeine did not reduce the germination of rice seeds under field conditions. We attribute

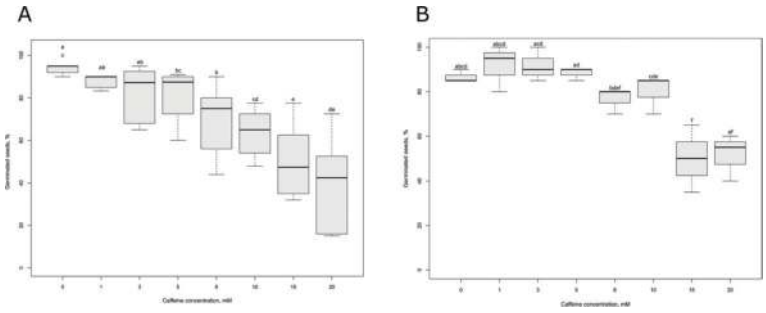

Figure 2. Effect of caffeine on seed germination of (A) lettuce cv. Queen of May and (B) tomato cv. Betalux. Germinated lettuce and tomato seeds were counted after 3 or 7 days of incubation, respectively.

	Cabbage cv. First harvest				**Tomato cv. Baron**				**Tomato cv. Betalux**			
Caffeine (mM)	0	1	5	8	0	1	5	8	0	1	5	8
14 days												
Growth and development	1U	2U	3P	4DW	1U	2U	5	5	1U	2U	3DW	4W
30 days												
Growth and development	1U	2DP	3DW	4W	1U	2DP	5	5	1 U	1DS	3DW	4W

Growth rate grading: 1—normal, 2—slower, 3—slow, 4—no growth, 5—no sprouted seeds. Development grading: U—uniform plant growth, D—darkening of the leaves, W—wilting, P—weaker plants, S—shed leaves. Photographs show growth and development of the representative plant for each treatment.

Table 2. Impact of caffeine on plant germination, growth and development *in vitro*.

this conclusion to ample water conditions required for rice cultivation that diminished the local concentration of caffeine.

Caffeine also impeded the germination, subsequent growth, and development of cabbage and tomato plants cultured on ½ MS medium (**Table 2**). Supplementation of the medium with caffeine in higher concentrations than 5 mM resulted in complete growth impairment of the tested plants (**Table 2**). In the case of plants growing on the 1 mM caffeine-enriched medium, they were weaker, exhibited slower growth rate, and certain darkening of the leaves after 30 days of incubation.

3.4. Effect of caffeine treatment on *in vitro*-grown and soil-grown plants

MS medium containing caffeine at concentrations higher than 5 mM completely inhibited *in vitro* regeneration of potato explants (cv. LB-6 and LB-12) (**Table 3**). Even application of 1 mM caffeine resulted in shorter potato plants of 5.3 ± 2.5 cm, in comparison with 10.7 ± 2.9 cm high controls. Similar pattern was shown in research on *Oryza sativa* L. by Smyth [24] who reported 2.5 mM caffeine suppression of shoot elongation by 50% and root elongation by 90%. Also in mung bean (*Phaseolus aureus*), Batish et al. [25] reported that caffeine reduced root number and length produced by hypocotyl cuttings.

Contrarily, spraying with 0, 1, 5, or 8 mM caffeine cabbage, lettuce, and tomato plants grown in soil did not significantly affect their growth or development as expressed by the plant heights measured after 6 weeks post planting (**Table 3**). Likewise, watering with 0, 1, 5, and 8 mM caffeine of lettuce and tomato plants grown in soil did not affect their heights that were

Plant	Plants heights (cm)			
	Caffeine concentration (mM)			
	0	1	5	8
Potato[1]	10.7 ± 2.9	5.3 ± 2.5	NG	NG
Tomato[2]	3.71 ± 0.62	3.33 ± 0.59	3.38 ± 0.53	3.26 ± 0.75
Cabbage[2]	6.56 ± 1.07	6.47 ± 1.07	6.32 ± 1.47	6.75 ± 1.13
Lettuce[2]	8.04 ± 1.40	8.58 ± 2.00	8.00 ± 1.21	8.36 ± 1.97
Tomato[3]	10.16 ± 1.53	10.23 ± 1.87	8.94 ± 1.83	8.46 ± 0.95
Lettuce[3]	10.35 ± 0.68	10.77 ± 1.50	9.88 ± 0.82	10.18 ± 1.00

[1]Micropropagation: Plants were grown on MS medium containing caffeine. Plants heights were measured after 6 weeks of incubation at 24°C (16/8 h light/dark photoperiod).

[2]Spraying: The seeds were germinated on moistened Whatman paper. After 2 weeks, they were planted in pots with autoclaved soil. Plants were grown at 20°C (16/8 h light/dark photoperiod) and were watered every 3 days. After 10 days, they were sprayed (10 ml per pot) with an aqueous solution containing caffeine. The caffeine was applied seven times over 6 weeks before the plant heights were measured.

[3]Watering: Seeds were planted in autoclaved soil. The plants were grown at 20°C (16/8 h light/dark photoperiod). Each pot was watered every 3 days with 30 ml of caffeine solution. After 4 weeks, plant heights were measured.

NG—no growth. Values are means ± SD.

Table 3. Effect of caffeine treatment on the heights of potato, tomato, cabbage, and lettuce plants.

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measured after 4 weeks of continuous growth (**Table 3**). This corresponds with Hollingsworth et al. [4] stating that 2% caffeine caused no phytotoxicity symptoms when it was sprayed on four varieties of 4-week-old lettuce plants growing in the greenhouse. They also observed no lesions on leaves or roots of any of the oncidium orchids. The only serious symptoms like yellowing of the leaves followed by necrosis appeared after several days on excised leaves of lettuce and cabbage after being dipped in caffeine solutions ranging from 0.5 to 2.0% [4].

3.5. Caffeine accumulation in plant tissue

HPLC analysis revealed that caffeine is accumulated in plants that have been treated with this compound (**Table 4**). The accumulation of caffeine was much greater if the plants had been exposed to caffeine on Whatman paper or on MS medium rather than in soil (**Table 4**). Interestingly, the amount of caffeine accumulated in tomato leaf tissue was much higher than in the stem or root tissues. Contrarily, lettuce leaves did not exhibit higher caffeine accumulation level than the corresponding sprouts (**Table 4**). In conclusion, the level of caffeine accumulation depends strongly on caffeine application method and varied between the investigated plant organs. The latter observation corresponds with unequal distribution of caffeine within plant species capable of synthesizing caffeine. For example, *Camellia sinensis* var. *sinensis* contains 2.8% caffeine in its foliage, while *Coffea arabica* seedlings contain caffeine mainly in the leaves and cotyledons at concentrations ranging from 0.8 to 1.9%. Caffeine is absent, however, in roots and in older, brown parts of *C. arabica* shoots [26]. Besides, an interesting observation was reported by Bustos [27] that stated caffeine accumulation in aromatic herbs like sage or oregano when they were intercropped with coffee.

3.6. Impact of peeling and/or cooking on caffeine accumulation

The concentration of caffeine in dry potato tissue was determined after tubers were incubated in a 100 mM caffeine solution at room temperature without subsequent cooking or with the

Plant	Plant organ	Caffeine concentration in plant tissue (mg g^{-1})			
		Caffeine concentration in the medium			
		0 mM	1 mM	5 mM	8 mM
Tomato	Leaves[1]	0.0559	0.0756	0.3467	0.3906
	Stems[1]	0.0072	0.0069	0.0613	0.0214
	Roots[1]	0.0040	0.0666	0.0786	0.0334
Lettuce	Sprouts[2]	0.0001	0.2651	1.8485	2.9674
	Leaves[1]	0.0354	0.0188	0.1580	0.0708
Potato	Plants[3]	0.1865	1.1396	3.9577	2.5106

[1]Plants grown in soil at 20°C (16/8 h light/dark photoperiod).

[2]Seeds sprouted on Whatman paper at 24°C (16/8 h light/dark photoperiod).

[3]Plants grown *in vitro* on MS medium at 24°C (16/8 h light/dark photoperiod).

Table 4. Influence of application method on the accumulation of caffeine in plant tissue.

cooking (±prior peeling) at 100°C. The caffeine concentration in uncooked potatoes was higher in the peel than in the middle or transitional zone of the tuber (**Figure 3**). Also, subjecting potatoes to cooking significantly reduced the overall caffeine content in the tuber tissue. We observed that total caffeine concentration was the lowest when potatoes were peeled before cooking (**Figure 3**). Importantly, *Solanum tuberosum* L. cv. Irga was used in this study, but we suspect differences in effectiveness of caffeine washing during cooking between potato cultivars, because their pectins vary in branching, methylation, and acetylation level, which can have an effect on potent caffeine removal [28].

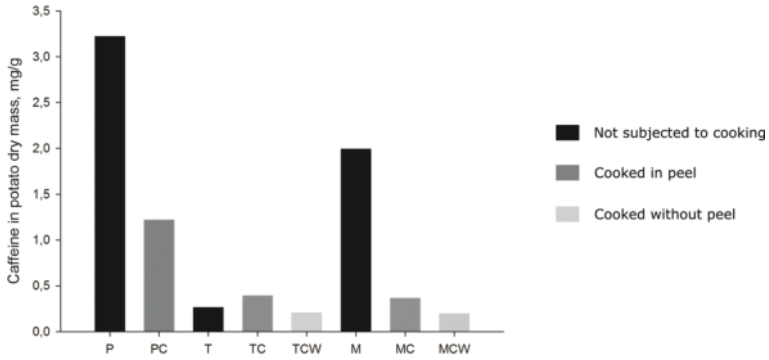

Figure 3. Caffeine concentration in the tissue of caffeine-treated potato tubers. Caffeine accumulated in the tissue originating from the following tuber zone after the indicated treatment: P—peel, without cooking; PC—peel, after cooking; T—transitional zone, without cooking; TC—transitional zone, after cooking; TCW—transitional zone, cooked without the peel; M—middle zone, without cooking; MC—middle zone, after cooking; MCW—middle zone, cooked without the peel.

4. Conclusions

World population is growing with an annual rate of 1.2%, meaning 77 million people per year [29], thus providing for food security and its safety appears crucial nowadays. Caffeine seems to be an attractive alternative for crop protection as it eradicates or repels molluscs, insects, frogs, birds, and phytopathogens [4–7, 12]. Even in the presence of compounds appearing in soil or plant extracts caffeine retained its inhibitory effect against Dsol, Pba, Pcc, Pst, Rsol, and Xcc, all mentioned by Mansfield et al. [30] in the list of top 10 plant pathogenic bacteria based on scientific and/or economic importance. So far, little is known about the possible ways to apply caffeine in agriculture. By now we have demonstrated that caffeine implementation on crop seeds could interfere with plant cell division and might inhibit the germination process. Thus, caffeine may be implemented before placing the potato seeds in storage, where inhibition of germination is an additional advantage. Importantly, watering and spraying of sprouts and the whole plants were proven not to interfere with further plant growth and development, so could be applied to agriculture in this form. Furthermore, our results showed that caffeine

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accumulated mainly in the peel of potato tuber and cooking significantly reduced the final caffeine content in all the tuber zones (especially while potatoes were peeled prior to thermal treatment).

As caffeine is obtained in commercial quantities by synthesis or as a by-product of the decaffeination process, the cost of the proposed treatment would not be high. Avery et al. calculated that rice treatment with 1% caffeine would cost the producers about 4$ ha^{-1} [7]. Not without importance is the fact that caffeine is readily soluble in water, which prevents its environmental accumulation. Moreover, caffeine is a common food additive of generally regarded as safe (GRAS) status, ingested directly in beverages such as tea or coffee throughout the world and even now it remains the fourth most frequently detected organic wastewater contaminant in the U.S. streams [31].

In conclusion, we think caffeine as a natural compound could be implemented effectively in agriculture in order to protect economically important crops and ornamentals from plant pathogenic bacteria.

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