ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 3/2015
605–612
http://dx.doi.org/10.18388/abp.2015_1092

Regular paper

Antibacterial activity of caffeine against plant pathogenic bacteria

Wojciech Sledz✉, Emilia Los, Agnieszka Paczek, Jacek Rischka, Agata Motyka, Sabina Zoledowska, Jacek Piosik and Ewa Lojkowska

Laboratory of Plant Protection and Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdańsk, Poland

The objective of the present study was to evaluate the antibacterial properties of a plant secondary metabolite — caffeine. Caffeine is present in over 100 plant species. Antibacterial activity of caffeine was examined against the following plant-pathogenic bacteria: *Ralstonia solanacearum* (Rsol), *Clavibacter michiganesis* subsp. *sepedonicus* (Cms), *Dickeya solani* (Dsol), *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pseudomonas syringae* pv. *tomato* (Pst), and *Xanthomonas campestris* subsp. *campestris* (Xcc). MIC and MBC values ranged from 5 to 20 mM and from 43 to 100 mM, respectively. Caffeine increased the bacterial generation time of all tested species and caused changes in cell morphology. The influence of caffeine on the synthesis of DNA, RNA and proteins was investigated in cultures of plant pathogenic bacteria with labelled precursors: [^{3}H]thymidine, [^{3}H]uridine or ^{14}C leucine, respectively. RNA biosynthesis was more affected than DNA or protein biosynthesis in bacterial cells treated with caffeine. Treatment of Pba with caffeine for 336 h did not induce resistance to this compound. Caffeine application reduced disease symptoms caused by Dsol on chicory leaves, potato slices, and whole potato tubers. The data presented indicate caffeine as a potential tool for the control of diseases caused by plant-pathogenic bacteria, especially under storage conditions.

Key words: antibacterial activity, plant secondary metabolites, *Ralstonia solanacearum*, *Clavibacter michiganesis* subsp. *sepedonicus*, *Dickeya solani*, *Pectobacterium atrosepticum*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* subsp. *campestris*

Received: 26 June, 2015; revised: 07 August, 2015; accepted: 10 August, 2015; available on-line: 26 August, 2015

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in nearly 100 plant species (Ashihara, 2006). Its main natural sources are tea (*Camellia sinensis* L.), coffee (*Coffea arabica* L.), cocoa (*Theobroma cacao*), and maté (*Ilex paraguariensis*). Caffeine's effects on human health have been broadly studied (Cano-Marquina *et al.,* 2013). Besides that, its antimicrobial properties have been examined against: human pathogens like *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*, constituents of natural human microflora such as *Escherichia coli*, *Streptococcus oralis*, and *Propionibacterium acnes*, but also *Pseudomonas fluorescens* and *Bacillus subtilis* that are present in terrestrial and aquatic habitats (Raj & Dhala, 1965; Ramanaviciene *et al.,* 2003; Cogo *et al.,* 2008, Mohammed & Al-Bayati, 2009). Researchers have also investigated the use of caffeine as a natural pesticide against insects, frogs, and slugs (Nathanson, 1984; Hollingsworth *et al.,* 2002). To our knowledge, the antibacterial activity of caffeine has been tested only against one phytopathogenic bacterium: *Pseudomonas syringae* pv. *glycinea* (Kim & Sano, 2008).

The objective of the present study was to evaluate antibacterial properties of caffeine against plant-pathogenic bacteria from the following species/subspecies: *Ralstonia solanacearum* (Rsol), *Dickeya solani* (Dsol), *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pseudomonas syringae* patovars, *Xanthomonas campestris* patovars and *Clavibacter michiganesis* subsp *sepedonicus* (Cms). All of the above-mentioned species, except for *Clavibacter michiganesis* subsp. *sepedonicus,* are present in the list of top ten plant-pathogenic bacteria based on their scientific and economic importance, proposed by Mansfield *et al.* (2012). It is worth to highlight that the control of diseases caused by the listed pathogens is difficult due to limited efficacy of biological and chemical agents and restricted use of antibiotics (Czajkowski *et al.,* 2011). According to data obtained in the current study, antibacterial activity of plant secondary metabolite caffeine could be implemented against plant pathogenic bacteria in crop protection worldwide.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains of the investigated plant-pathogenic bacteria originated from the collection of the Department of Plant Protection and Biotechnology Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (IFB UG & MUG). All strains used in this study, implemented media and growth conditions are listed in Table 1.

Effect of caffeine on the growth of tested bacteria. Bacterial growth curves in media containing 0, 1, 3, 5, 8, 10, 15 or 20 mM of caffeine were plotted on the basis of OD_{580} measurements conducted every 60 min for 24 h (or for 48 h in the case of Cms) in 24-well

✉e-mail: wojciech.sledz@biotech.ug.edu.pl

Abbreviations: Cms, *Clavibacter michiganesis* subsp. *sepedonicus;* Dsol, *Dickeya solani*; IFB UG & MUG, Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk; LB, lysogeny broth; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; PR-1a, pathogenesis-related protein 1a; Pba, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; Pst, *Pseudomonas syringae* pv. *tomato;* PI-II, proteinase inhibitor II; Rsol, *Ralstonia solanacearum*; dTTP, deoxythymidine triphosphate; Xcc, *Xanthomonas campestris* subsp. *campestris*

Table 1. Plant-pathogenic bacteria used in this study and their growth conditions

Bacterium (abbreviation), strain	Growth temperature (°C)	Solid medium used	Liquid medium used
Ralstonia solanacearum (Rsol), LMG 2294	28	TZC + A	TZC
Clavibacter michiganesis subsp. *sepedonicus* (Cms), LMG 2889	21	NCP-88 + A	NCP-88
Dickeya solani (Dsol), IFB 0099	28	LA	LB
Pectobacterium atrosepticum (Pba), SCRI 1043	28	LA	LB
Pectobacterium carotovorum subsp. *carotovorum* (Pcc), SCRI 180	28	LA	LB
Pseudomonas syringae pv. *tomato* (Pst), LMG 5093	28	LA	LB
Xanthomonas campestris pv. *campestris* (Xcc), LMG 582	28	GF + A	GF

LMG, BCCM/LMG Bacteria Collection, Gent, Belgium; SCRI, Scottish Crop Research Institute, Dundee, Scotland; IFB, Intercollegiate Faculty of Biotechnology UG&MUG Bacteria Collection, Gdansk, Poland; LB, Lysogeny Broth medium (Bertani, 1951); LA medium, LB + A; GF, Growth Factor medium (Agarwal *et al*., 1989); NCP-88 medium (De la Cruz *et al*., 1992); TZC + A medium, 2,3,5-triphenyl tetrazolium chloride (French *et al*., 1995); A, agar 15 g l^{-1}

plates (Sarstedt, Nuembrecht, Germany). Bacteria were incubated at their optimal growth temperature (Table 1) with shaking at 250 rpm. Generation times, defined as the time in which OD_{580} of bacterial cultures increased from 0.1 to 0.2, were calculated from the growth curves. Experiments were performed in triplicate.

Evaluation of MIC and MBC values of caffeine. Bacterial strains listed in Table 1 had been incubated under optimal conditions for 24 h (48 h for Cms) and the density of each culture was adjusted to 0.5 McFarland scale (approx. 10^8 CFU×ml^{-1}). Obtained bacterial suspensions were diluted 100 times, and aliquots of 100 µl were transferred to wells in 96-well microplates (Sarstedt). Bacteria were subjected to aqueous solutions containing caffeine at concentrations ranging from 0 to 100 mM. After 24 h incubation at 28°C (48 h at 21°C for Cms), the lowest concentration of caffeine inhibiting bacterial growth was designated as the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) was defined as the lowest concentration of caffeine that eliminated 99.9% of bacterial cells within 24 h (Thornsberry, 1991; Szpitter *et al*., 2014). Experiments were performed in triplicate.

Effect of caffeine on the morphology of bacterial cells. Bacterial strains were cultured in media with 8 mM caffeine for 24 h (36 h for Cms) at the optimal temperature with shaking (200 rpm). A 5 µl aliquot of each culture was placed on the surface of a 300-mesh copper grid (Sigma-Aldrich) and incubated at room temperature for 3 min. After excess fluid was removed, bacteria were stained for 30 s with 1% uranyl acetate (VWR International, Radnor, USA). The samples were observed with a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands) at 2200× magnification. The image was analyzed with ITEM software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

Effect of caffeine on DNA replication, RNA and protein biosynthesis in bacterial cells. The procedures of Wegrzyn *et al.* (1991) and Szpitter *et al.* (2014) were followed. Briefly, Dsol, Rsol, Pba, Pcc, Cms, Pst, Xcc suspensions were adjusted to OD_{580} = 0.1. Caffeine solution was added to obtain a final concentration of 5 mM. Bacteria not treated with caffeine were used as a control. The preparations were then supplemented with radioactive isotopes, i.e., with 5 µCi×ml^{-1} [^{3}H] thymidine (Hartmann Analytic, Braunschweig, Germany), 5 µCi×ml^{-1} [^{3}H] uridine (Hartmann Analytic), or 2 µCi×ml^{-1} [^{14}C] leucine (Hartmann Analytic) for DNA, RNA, and protein biosynthesis analyses, respectively. Obtained cultures were incubated at optimal temperatures, and 50-µl samples were collected every 15 min and placed on Whatman filter papers (Sigma-Aldrich) for 10 min with 10% trichloroacetic acid (Sigma-Aldrich) and for 10 min with 5% trichloroacetic acid. Then, the filters were washed with 96% ethanol and dried. The radioactivity in the filters was measured with the LS3133P scintillation counter (Beckman-Coulter, Pasadena, USA).

Examination of Pba ability to become caffeine resistant. Pba was grown in LB medium with 8 mM caffeine for 336 h (bacteria were passaged to fresh medium with caffeine every 24 h). To evaluate whether Pba cells acquired resistance to caffeine, they were transferred to LB medium supplemented with 0, 8, or 20 mM caffeine. Pba not pretreated with caffeine served as a control. Bacterial growth in the mentioned media was monitored for 24 h. The experiment was performed in triplicate.

Influence of caffeine on plant tissue maceration by Dsol. Chicory leaves (five leaves per replicate) were sprayed with 25 or 100 mM caffeine solutions. A sterile knife was used to gently cut the leaves across the vascular bundles (one cut per leaf), and the cuts were inoculated with 20 µl of 10^6 CFU×ml^{-1} of Dsol. Leaves were sealed in plastic bags containing moist paper towels. After 24 h at 28°C, the diameters of necrotic spots were measured. The experiment was performed twice.

Potato tubers (cv. Irga) were surface-sterilized with 5% calcium hypochlorite, washed, sprayed with 70% ethanol, and cut into 1-cm-thick slices. Three 0.5-cm-diameter cavities were formed on the surface of each slice, and then each cavity was treated with 50 µl of 10^8 CFU×ml^{-1} of Dsol or 50 µl of 10^8 CFU×ml^{-1} of Dsol in 100 mM caffeine solution. Water was used as a control. After 24 h at 28°C, the diameters of macerated tissue were measured. Each combination of treatments was represented by four replicate potato slices, and the experiment was performed twice.

The effect of caffeine on Dsol-induced disease severity in intact potato tubers was also evaluated. Tubers (cv. Irga) were surface-sterilized with 5% calcium hypochlorite, washed, and sprayed with 70% ethanol. Three tubers were sealed in one plastic bag with 10 ml of one of the following suspensions: Dsol at 10^8 CFU×ml^{-1} in Ringers solution and Dsol at 10^8 CFU×ml^{-1} plus 100 mM caffeine. Sterile water was used as a control. Each treatment was represented by 3 replicate bags. After 14 days at 28°C, the tubers were visually assessed for rotting symptoms. The disease severity was scored in a range from 0 to 3 at the end of treatment. Experiments were performed twice. Maceration rate: 0 — no maceration; 1 — very low; 2 — intermediate; 3 — high.

Table 2. MIC and MBC values of caffeine towards plant-pathogenic bacteria tested

Bacterial species	MIC of caffeine (mM)	MBC of caffeine (mM)
Dickeya solani	18.3 ± 2.9	80.0 ± 8.7
Ralstonia solanacearum	20 ± 0.1	43.3 ± 5.8
Clavibacter michiganensis subsp. *sepedonicus*	10 ± 0.1	80.0 ± 0.1
Pectobacterium atrosepticum	8.7 ± 1.2	100.0 ± 0.1
Pectobacterium carotovorum subsp. *carotovorum*	9.0 ± 1.2	100.0 ± 0.1
Pseudomonas syringae pv. *tomato*	5.0 ± 0.1	43.3 ± 5.77
Xanthomonas campestris subsp. *campestris*	10.0 ± 0.1	75.0 ± 0.1

Abbreviations for bacterial species are explained in Table 1. Values are means ± SE

The effect of caffeine on natural microbial community on the surface of potato tubers was also investigated. Unsterilized potato tubers (cv. Irga) were sealed in plastic bags (three tubers per bag) containing 15 ml of 100 mM caffeine or 15 ml of sterile H_2O. Bags with tubers (three replicate bags per treatment) were stored at 4°C for about 60 days, when rotting symptoms first became evident. Severity of developed disease symptoms was visually assessed and scored in a range from 0 to 3 at the end of treatment. Maceration rate: 0 — no maceration; 1 — very low; 2 — intermediate; 3 — high. Experiments were performed twice.

Statistical analysis. Caffeine impact on DNA, RNA and protein synthesis in bacteria was assessed with Levene's test ($p<0.05$) and Student's t-test ($p<0.05$). The effect of caffeine on disease severity was evaluated with X^2 test.

RESULTS

Antibacterial activity of caffeine against phytopathogens

The antibacterial activity of caffeine was tested against bacteria belonging to seven species/subspecies. Caffeine exhibited antibacterial properties against all tested plant pathogenic bacteria. This compound suppressed growth of all tested bacteria in a dose-dependent manner (Fig. 1).

MIC and MBC values of caffeine against Rsol, Cms, Dsol, Pba, Pcc, Pst, and Xcc are shown in Table 2. MIC values ranged from 5 to 20 mM. MIC value was the lowest for Pst and the highest for Rsol. MBC values ranged from 43 to 100 mM. MBC values were the lowest for Pst and Rsol and the highest for Pba and Pcc.

Bacterial generation time of all seven tested species increased as the concentration of caffeine applied to the medium was elevated (Fig. 2).

Effects of caffeine on bacterial cell morphology

Caffeine induced abnormal changes in bacterial cell morphology. When grown in a medium containing 8 mM caffeine, the Dsol cells were elongated (Fig. 3AB). Pba and Pst cells displayed a similar elongation level to Dsol (not shown), while Xcc cells formed long chains (Fig. 3CD). On the other hand, Cms cells exhibited abnormal morphology and they were probably lysed (Fig. 3EF), whereas the morphology of Pcc and Rsol cells was unaffected by 8 mM caffeine (not shown).

Influence of caffeine on DNA, RNA and protein synthesis in tested bacteria

The influence of caffeine on the DNA replication, RNA and protein synthesis was estimated by measuring the incorporation of radioactive precursors according to a previously described procedure (Wegrzyn *et al.*, 1991). To learn more about caffeine mechanism of action against plant pathogenic bacteria the effect of caffeine on basic cellular biochemical processes was monitored. DNA replication tended to be suppressed by a 90-min exposure to 5 mM caffeine in the case of Dsol, Rsol, and Pba but not in the case of Pcc, Pst, or Xcc (Fig. 4). RNA synthesis was significantly reduced by 5 mM caffeine even after just 15 min of treatment of Dsol, Pba, and Pcc (Fig. 4). Protein biosynthesis was not significantly affected by exposure to caffeine for up to 120 min (Fig. 4).

Examination of Pba ability to become caffeine resistant

The ability of caffeine to induce resistance in bacterial cells was studied. 8 mM caffeine pretreatment for 336 h did not result in developing resistance to this compound by Pba cells. These bacteria were equally susceptible to subsequent caffeine application (Fig. 5).

Effect of caffeine on plant tissue maceration caused by Dsol

Application of caffeine at 25 mM concentration resulted in reduction of chicory tissue maceration level caused by Dsol (Table 3). Application of 50 mM or 100 mM solution of caffeine completely inhibited the maceration of chicory leaves (Table 3, Fig. 6). Potato tuber slices inoculated with Dsol and treated with caffeine also indicated reduction in tissue maceration level when the caffeine solution was applied. 100 mM caffeine supplementation completely inhibited maceration of potato slices by Dsol, while in the case of slices untreated with caffeine the diameter of macerated tissue was about 15 mm (Table 3, Fig. 6).

Concerning results obtained for whole, sterile potato tubers artificially-inoculated with 50 µl of 10^8 CFU×ml^{-1} of Dsol, application of 100 mM caffeine and incubation for 14 days at 28°C under anaerobic conditions promoting tissue maceration, resulted in suppression of the disease symptom development (Table 3, Fig. 6).

Moreover, potato tissue maceration (wet and/or dry rot), that appeared on intact non-sterilized tubers incubated in plastic bags for 60 days at 4°C with the solution of 100 mM caffeine, was also much less se-

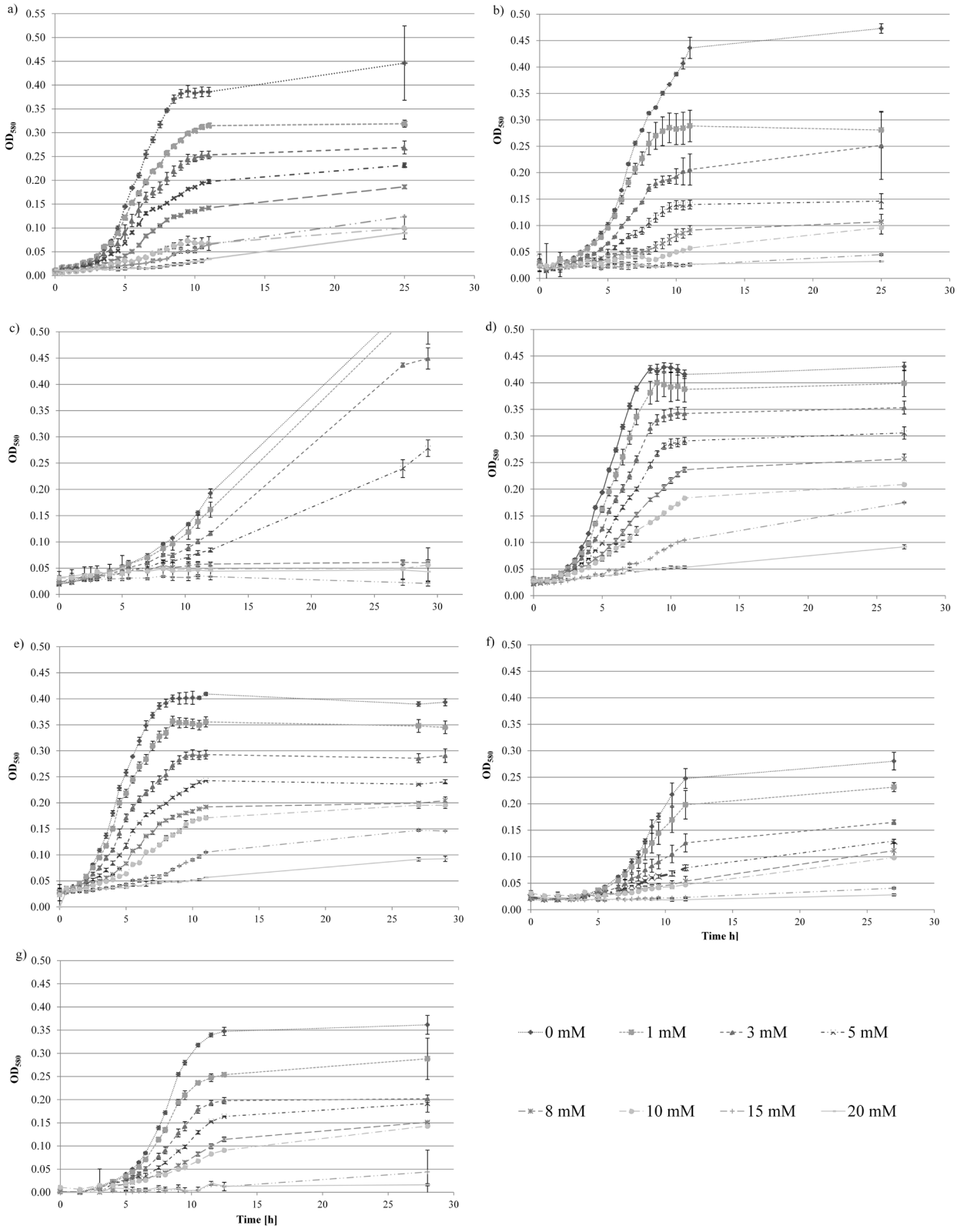

Figure 1. Effect of different concentrations of caffeine on growth of bacteria:
(**a**) *Dickeya solani*, (**b**) *Ralstonia solanacearum*, (**c**) *Clavibacter michiganensis* subsp. *sepedonicus*, (**d**) *Pectobacterium atrosepticum*, (**e**) *Pectobacterium carotovorum* subsp. *carotovorum*, (**f**) *Pseudomonas syringae* pv. *tomato*, (**g**) *Xanthomonas campestris* subsp. *campestris*.

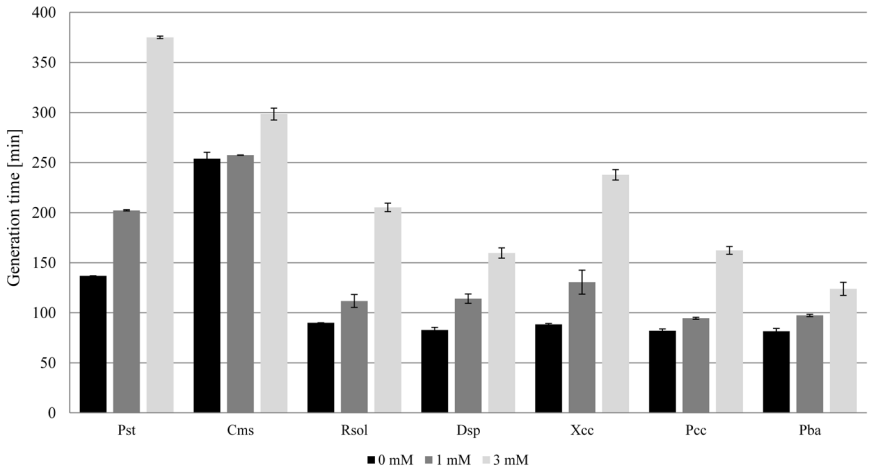

Figure 2. Generation time of bacteria as affected by caffeine concentration in the media.

vere than in the case of tubers incubated without caffeine under the same conditions (Table 3, Fig. 6).

DISCUSSION

Plant secondary metabolites have been widely used in pharmaceutical, food, chemical, textile, and agricultural industries (Croteau *et al.*, 2000). Because these natural molecules are the products of coherent evolution, researchers have been searching among them for active agents inhibiting crop pathogens (Zheng *et al.*, 2010; Szpitter *et al.*, 2014). The functions of caffeine in plants that produce this compound are still unclear, but two hypotheses have been presented: one is that caffeine protects plants against pests, and the other is that caffeine prevents seed germination near parent plants (Ashihara *et al.*, 2008). On the basis of data presented in this work, we postulate the first hypothesis to be true.

All seven of the plant-pathogenic bacteria included in this study were sensitive to caffeine, with MIC and MBC values ranging from 5 to 20 mM and from 43 to 100 mM, respectively. These results are consistent with data concerning the antibacterial activity of caffeine against human pathogens or common free-living microbes. Raj & Dhala (1965), for example, reported that 5000 $\mu g \times ml^{-1}$ (about 25 mM) caffeine inhibited many different free-living bacteria as well as human pathogens. Ramanaviciene *et al.* (2003) stated that a 1% (about 50 mM) caffeine solution substantially reduced the growth rate of *E. coli* and that of an ordinary constituent of crop microflora – *P. fluorescens*. Although Cogo *et al.* (2008) reported that caffeine did not alter the growth of human oral microbiota, the highest concentration in their treatment was 400 $\mu g \times ml^{-1}$ (about 2 mM).

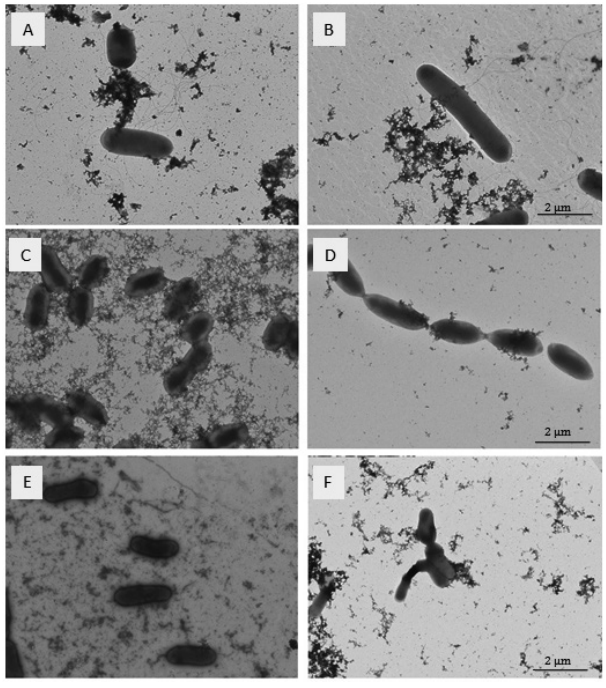

Figure 3. Morphology of bacteria growing in media containing 8 mM caffeine in comparison to the controls:
(**A**) *Dickeya solani* in 0 mM caffeine, (**B**) *Dickeya solani* in 8 mM caffeine, (**C**) *Xanthomonas campestris* subsp. *campestris* in 0 mM caffeine, (**D**) *Xanthomonas campestris* subsp. *campestris* in 8 mM caffeine, (**E**) *Clavibacter michiganensis* subsp. *sepedonicus* in 0 mM caffeine, (**F**) *Clavibacter michiganensis* subsp. *sepedonicus* in 8 mM caffeine.

To our knowledge, the only species of plant pathogenic bacteria that has been tested before for the susceptibility to caffeine is *P. syringae* pv. *glycinea*. Kim & Sano (2008) found that 2 mM caffeine completely suppressed the growth of *P. syringae* pv. *glycinea*. In our study, 5 mM caffeine was needed to inhibit the growth of its close relative, *P. syringae* pv. *tomato*.

The data gathered indicated that 20 mM caffeine solution can effectively inhibit growth of all tested plant pathogenic bacteria. Our findings also provide insight into the mechanisms by which caffeine inhibits the growth of plant-pathogenic bacteria. Caffeine increased the bacterial generation time and influenced the rate of cell division. In addition, caffeine treatment changed Pba, Pst, and Dsol cell morphology and induced Xcc to form cell chains. This outcome is consistent with studies on *Aerobacter aerogenes* and *A. cloacae* (Raj & Dhala, 1965) and several *E. coli* strains (Sandlie *et al.*, 1980). In contrast, caffeine did not significantly change the morphology of Pcc or Rsol (not shown), which is in accordance with a report on *S. aureus* (Raj & Dhala, 1965).

Based on our data, caffeine affected RNA synthesis more than DNA replication or protein synthesis in the cells of plant-pathogenic bacteria. According to studies with *E. coli*, caffeine mainly affects DNA synthesis and more specifically affects the thymidine uptake, its conversion to dTTP, and the process of DNA replication itself. Additionally, caffeine has an influence on the following enzymes: purine nucleoside phosphorylase, DNA polymerase I, and uvrAuvrBuvrC endonuclease (Sandlie *et al.*, 1980). Lieb (1961) found that caffeine, in addition to reducing DNA replication, reduced the rate of RNA and protein synthesis. Similarly, Labbe & Nolan (1987) reported that

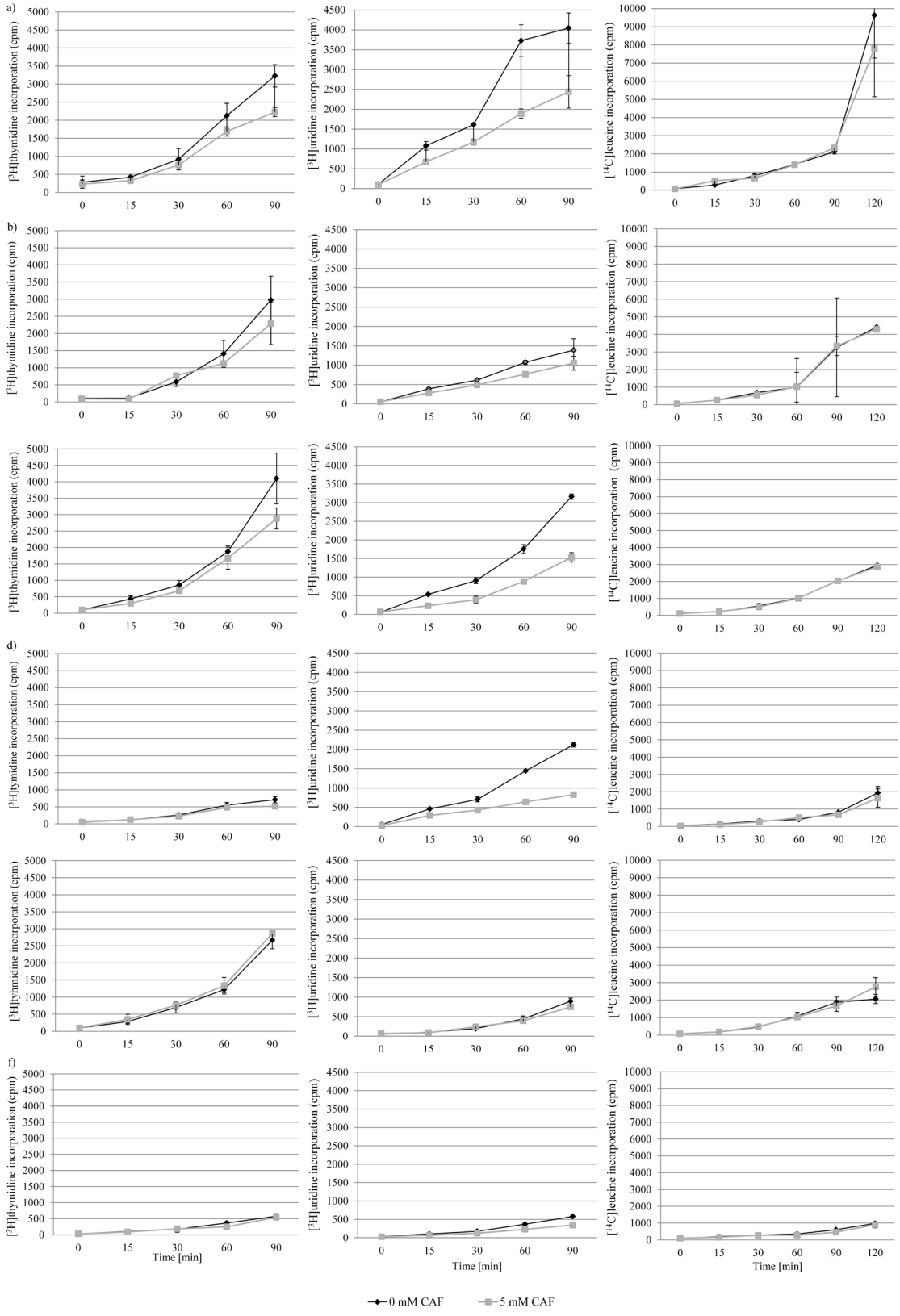

Figure 4. Effects of caffeine on DNA replication, RNA and protein synthesis:
(**a**) *Dickeya solani*, (**b**) *Ralstonia solanacearum*, (**c**) *Pectobacterium atrosepticum*, (**d**) *Pectobacterium carotovorum* subsp. *carotovorum*, (**e**) *Pseudomonas syringae* pv. *tomato*, and (**f**) *Xanthomonas campestris* subsp. *campestris*. Values are means ± SE. Statistical significance is as follows: $p<0.0001$ extremely significant (****), $0.0001<p<0.001$ extremely significant (***), $0.001<p<0.01$ very significant (**), $0.01<p<0.05$ significant (*).

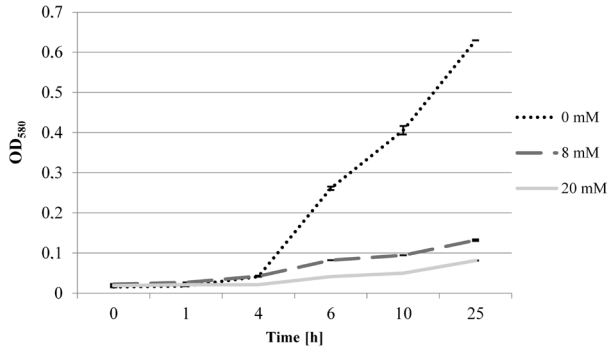

Figure 5. Growth of *Pectobacterium atrosepticum* as affected by 0 mM, 8 mM and 20 mM caffeine after 336 h of prior exposure to 8 mM caffeine.

caffeine inhibited the incorporation of [^{14}C]adenine, [^{14}C]thymidine, and ^{14}C-labeled amino acids in *Clostridium perfringens*. They also stated that [^{14}C]caffeine was not incorporated into the DNA strand. Additional research is needed to clarify the mechanism(s) by which caffeine inhibits plant-pathogens and other bacteria.

In plant tissue maceration assays performed on chicory leaves, potato slices and whole potato tubers (surface-sterilized and inoculated with Dsol or non-sterilized), treatment with caffeine solutions reduced disease symptoms caused by bacteria from the species *D. solani*. The antibacterial activity of caffeine was shown by Kim & Sano (2008) in transgenic tobacco plants that were capable of synthesizing caffeine and were highly resistant to *Pseudomonas syringae* pv. *glycinea*. They claimed that caffeine, whether applied exogenously or synthesized *in planta*, activates host defense by elevating the expression of the pathogenesis-related protein 1a (PR-1a) and proteinase inhibitor II (PI-II). In addition to inhibiting plant-pathogenic bacteria, caffeine might be useful for the control of other pests and as a repellent of frogs, snails, or birds in crops (Nathanson 1984; Hollingsworth *et al.*, 2002; Avery *et al.,* 2005).

In conclusion, caffeine's broad spectrum of activity against economically significant plant-pathogenic bacteria suggests potential application of this compound in crop protection against phytopathogens. Further work is needed to determine whether the presented benefits of caffeine can be adopted for the storage or field conditions.

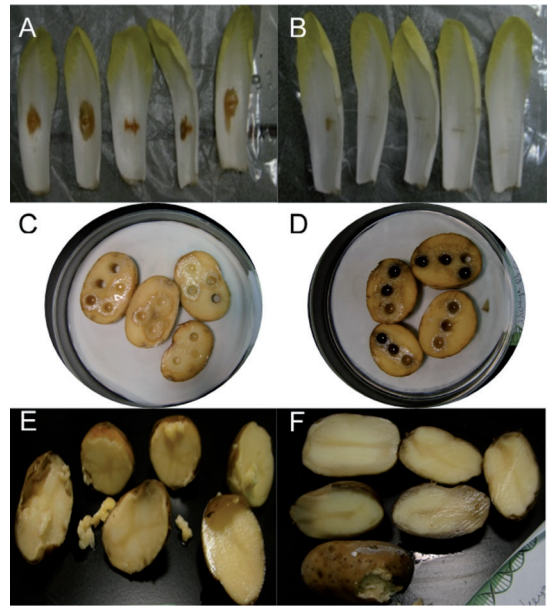

Figure 6. Influence of 100 mM caffeine on plant tissue maceration caused by *Dickeya solani*.
Chicory leaves inoculated with 20 µl of 10^6 CFU ml^{-1} of *D. solani* suspension and incubated for 24 h at 28°C. (**A**) Not treated with caffeine. (**B**) Treatment with 100 mM caffeine. Potato tuber slices inoculated with 50 µl of 10^8 CFU×ml^{-1} of *D. solani* suspension and incubated for 24 h at 28°C. (**C**) Not treated with caffeine. (**D**) Treatment with 100 mM caffeine. Sterilized potato tubers were incubated in sealed plastic bags with 10 ml of 10^8 CFU×ml^{-1} of of *D. solani* suspension and incubated for 14 days at 28°C. (**E**) Not treated with caffeine. (**F**) Treatment with 100 mM caffeine.

Acknowledgements

We thank the research groups of Professor Bogdan Banecki and Professor Grzegorz Wegrzyn for valuable support in HPLC analyses and the radioactivity assay, respectively.

This study and publication was funded by The National Science Center in Poland *via* grant number NN 310150535 (MNiSzW 1505/B/P01/2008/35) to Wojciech Sledz.

REFERENCES

Agarwal PC, Mortensen CN, Mathur SB (1989) Seedborne disease and seed health testing of rice. In *Technical Bulletin* **3**. *Phytopathol Pap* **30**.

Table 3. Effect of caffeine on maceration of chicory leaves and potato tubers by *Dickeya solani*.

	Inoculation with suspension of *Dickeya solani*		
Treatment Plants	H_2O	Caffeine 25 mM	Caffeine 100 mM
[a]Wounded chicory leaves	8.6 ± 5.2	2.14 ± 2.1	0
[b]Potato slices	14.6 ± 2.5	Nd	0
[c]Inoculated potato tubers	2	Nd	1
[d]Non-inoculated potato tubers	3	Nd	1

[a]Chicory leaves were inoculated with 20 µl of *D. solani* suspension containing 10^6 CFU×ml^{-1}. Diameter of macerated tissue was measured after 24 h incubation at 28°C. [b]Potato tuber slices were inoculated with 50 µl of *D. solani* suspension containing 10^8 CFU ml^{-1}. Diameter of macerated tissue was measured after 24 h incubation at 28°C. [c]Sterilized potato tubers were incubated in sealed plastic bags with 10 ml of water, 10 ml of *D. solani* suspension containing 10^8 CFU×ml^{-1} and 10 ml of *D. solani* suspension containing 10^8 CFU×ml^{-1} together with 100 mM caffeine solution. Tissue maceration was evaluated after 14 day incubation at 28°C. Severity of disease symptoms was scored in a range from 0 to 3 at the end of experiment. Maceration rate: 0 — no maceration; 1 — very low; 2 — intermediate; 3 — high. [d]Non-sterilized potato tubers were incubated in sealed plastic bags with 15 ml of water or 15 ml of 100 mM caffeine. Tissue maceration was evaluated after 60 days incubation at 4°C. Severity of disease symptoms was graded as a range from 0 to 3 at the end of experiment. Maceration rate: 0 — no maceration; 1 — very low; 2 — intermediate; 3 — high. Values are means ±SE. Nd, not determinated.

Danish Government Institute of Seed Pathology for Developing Countries and CAB International Mycological Institute.
Ashihara H (2006) Metabolism of alkaloids in coffee plants. *Brazil J Plant Physiol* **18**: 1–8. http://dx.doi.org/10.1590/S1677-04202006000100001.
Ashihara H, Sano H, Crozier A (2008) Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochem* **69**: 841–856. doi:10.1016/j.phytochem.2007.10.029.
Avery ML, Werner SJ, Cummings JL, Humphrey JS, Milleson MP, Carlson J C, Primus TM, Goodall MJ (2005) Caffeine for reducing bird damage to newly seeded rice. *Crop Prot* **24**: 651–657. doi:10.1016/j.cropro.2004.11.009.
Bertani G (1951) Studies on lysogenesis I: the mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293-300.
Cano-Marquina A, Tarín JJ, Cano A (2013) The impact of coffee on health. *Maturitas* **75**: 7–21. doi:10.1016/j.maturitas.2013.02.002.
Cogo K, Montan MF, Bergamaschi CdeC, Andrade ED, Rosalen PL, Groppo FC (2008) *In vitro* evaluation of the effect of nicotine, cotinine and caffeine on oral microorganisms. *Can J Microbiol* **54**: 501–508. doi:0.1139/W08-032.
Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In *Biochemistry and Molecular Biology of Plants*: 1250–1318. American Society of Plant Physiologists. Rockville, MD.
Czajkowski R, Perombelon MCM, van Veen JA, van der Wolf JM (2011) Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. *Plant Pathol* **60**: 999–1013. doi: 10.1111/j.1365-3059.2011.02470.x.
De la Cruz AR, Wiese MV, Schaad NW (1992) A semiselective agar medium for isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from potato tissues. *Plant Dis* **76**: 830–834.
French ER, Gutarra L, Aley P, Elphinstone J (1995) Culture media for *Pseudomonas solanacearum* isolation, identification and maintenance. *Fitopatol* **30**: 126–130.
Hollingsworth RG, Armstrong JW, Campbell E (2002) Pest Control: Caffeine as a repellent for slugs and snails. *Nature* **417**: 915–916. doi:10.1038/417915a.
Kim YS, Sano H (2008) Pathogen resistance of transgenic tobacco plants producing caffeine. *Phytochemistry* **69**: 882–888. doi:10.1016/j.phytochem.2007.10.021.
Labbe RG, Nolan LL (1987) Inhibition of macromolecular synthesis by caffeine in *Clostridium perfringens*. *Can J Microbiol* **33**: 589–592. doi:10.1139/m87-102.
Lieb M (1961) Enhancement of ultraviolet-induced mutation in bacteria by caffeine. *Mol Gen Genet* **92**: 416–429. doi:10.1007/BF00890063.
Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol Plant Pathol* **13**: 614–629. doi: 10.1111/j.1364-3703.2012.00804.x.
Mohammed MJ, Al-Bayati FA (2009) Isolation, identification and purification of caffeine from *Coffea arabica* L. and *Camellia sinensis* L.: A combination antibacterial study. *Int J Green Pharm* **3**: 52–57. doi: 10.4103/0973-8258.49375.
Nathanson JA (1984) Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science* **226**: 184–187. doi: 10.1126/science.6207592.
Raj CVS, Dhala S (1965) Effect of naturally occurring xanthines on bacteria. I. Antimicrobial action and potentiating effect on antibiotic spectra. *Appl Microbiol* **13**: 432–436.
Ramanaviciene A, Mostovojus V, Bachmatova I, Ramanavicius A (2003) Anti-bacterial effect of caffeine on *Escherichia coli* and *Pseudomonas fluorescens*. *Acta Med Lit* **10**: 185–188.
Sandlie I, Solberg K, Kleppe K (1980) The effect of caffeine on cell growth and metabolism of thymidine in *Escherichia coli*. *Mutat Res* **73**: 29–41. doi:10.1016/0027-5107(80)90133-5.
Summers RM, Seffernick JL, Quandt EM, Yu CL, Barrick JE, Subramanian M V (2013) Caffeine junkie: an unprecedented glutathione S-transferase-dependent oxygenase required for caffeine degradation by *Pseudomonas putida* CBB5. *J Bacteriol* **195**: 3933–3939. doi:10.1128/JB.00585-13.
Szpitter A, Narajczyk M, Maciag-Dorszynska M, Wegrzyn G, Lojkowska E, Krolicka A (2014). Effect of *Dionaea muscipula* extract and plumbagin on maceration of potato tissue by *Pectobacterium atrosepticum*. *Ann App Biol* **164**: 404–414. doi: 10.1111/aab.12110.
Thornsberry C (1991) Antimicrobial susceptibility testing: general considerations. In *Manual of Clinical Microbiology*, pp 1059–1201. Washington DC, American Society for Microbiology.
Wegrzyn G, Kwasnik E, Taylor K (1991) Replication of λ plasmid in amino acid-starved strains of *Escherichia coli*. *Acta Biochim Pol* **38**: 181–186.
Zheng S, Lui Q, Zhang G, Wang H, Ng TB (2014) Purification and characterization of an antibacterial protein from dried fruiting bodies of the wild mushroom *Clitocybe sinopica*. *Acta Biochim Pol* **57**: 43–48.