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„Influence of endophytes on the production of phenolic compounds in cell suspension cultures of *Bergenia pacumbis* (Buch.-Ham. ex D.Don) C.Y.Wu & J.T.Pan“

verfasst von / submitted by

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List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
BAP	6-Benzylaminopurine
FDA	Fluorescein diacetate
HPLC	High performance liquid chromatography
ISR	Induced systemic resistance
JA	Jasmonic acid
LB	Lysogeny broth
MS	Murashige & Skoog medium
NAA	1-Naphthaleneacetic acid
PCTC	Plant cell and tissue culture(s)
SA	Salicylic acid
TBE	Tris, borate, EDTA
TFA	Trifluoroacetic acid
TSB	Tryptic soy broth

1. Abstract / Zusammenfassung

Abstract

The aim of this work was to investigate the general potential of endophytes, isolated from the plant *Bergenia pacumbis*, to influence the production of secondary metabolites (specifically the phenolic compounds arbutin and bergenin) in cell suspension cultures of *B. pacumbis*. It was therefore necessary to transfer callus lines, which were derived from the very plant during a previous project, from semi-solid to liquid medium, in order to achieve (optimally) single cell suspensions. After attempting various strategies to obtain suspensions, it was decided to use finely chopped cell aggregates. In a next step a growth curve of the used callus lines (BP 57 and BP 179) was established to determine the ideal time for elicitation. For this purpose, a non-invasive method (sc. the measurement of cell volume after sedimentation) was used. In parallel to the experiments with suspension cultures a part of the previously isolated bacterial endophytes was identified using 16S rDNA-sequencing, the results were also use to generate a phylogenetic tree. Five of these endophytes were chosen, in regards to their taxonomic diversity and potential to produce interesting secondary metabolites, for the subsequent elicitation. They were cultivated in LB- and TSB-medium and growth curves established. Finally autoclaved bacterial cells on the one hand, and sterile filtrated medium on the other hand was added to the plant suspension cultures at the estimated optimal time and incubated for two days. Subsequently cells and medium were separated, lyophilized and extractions performed. The extracts were finally analyzed by HPLC.

The results showed on the one hand, that in both lines neither arbutin nor bergenin were detectable to a reproducible extent in the cell suspension media. Furthermore no detectable amounts of arbutin were found in the cell extracts. In regards to the effects on the production of bergenin a strong dichotomy was apparent: while cell line BP 57 showed the expected statistically-significant increase after some treatments, almost all treatments of line BP 179 led to a significant decrease of bergenin content. Possible reasons for these unexpected results were discussed at the end of this thesis, however, further targeted research is necessary for a satisfying elucidation.

Zusammenfassung

Ziel der vorliegenden Arbeit war es, das grundsätzliche Potential von aus der Pflanze *Bergenia pacumbis* isolierten Endophyten, auf die Sekundärmetabolitproduktion (im Speziellen: die phenolischen Stoffe Arbutin und Bergenin) von Zellsuspensionskulturen einzuwirken, zu untersuchen. Dafür war es in einem ersten Schritt notwendig, Kalluslinien, die bereits in einem vorhergehenden Projekt aus ebendieser Pflanze gewonnen wurden, vom halbfesten in ein Flüssigmedium zu überführen, mit dem Ziel, möglichst feine Zellsuspensionen zu gewinnen. Nach Erprobung einiger Techniken wurden für diese Suspensionskulturen kleingehackte Zellaggregate verwendet. In einem nächsten Schritt wurde zur Ermittlung des optimalen Elicitierungszeitpunktes eine Wachstumskurve der zu verwendenden Zelllinien (BP 57 und BP 179) erstellt. Dabei wurde von einer nicht-invasiven Methode (Messung des Zellsedimentationsvolumens) Gebrauch gemacht. Parallel zu den Versuchen mit Suspensionskulturen wurden früher isolierte bakterielle Endophyten mittels 16S rDNA-Sequenzierung identifiziert, die Resultate wurden weiterhin zur Erstellung eines phylogenetischen Baums verwendet. Fünf Endophyten wurden in Hinblick auf ihre taxonomische Diversität und das Potential, interessante Sekundärmetaboliten zu produzieren, für die nachfolgende Elicitierung ausgewählt. Diese wurden in LB- und TSB-Medium kultiviert und Wachstumskurven erstellt. Es wurden schließlich einerseits die autoklavierten Bakterienzellen selbst, andererseits das sterilfiltrierte Medium den Suspensionskulturen zum ermittelten optimalen Zeitpunkt zugefügt und für zwei Tage inkubiert. Anschließend wurden die Kalluszellen und das Suspensionsmedium durch Abnutschen voneinander getrennt, lyophilisiert und Extraktionen vorgenommen. Die Extrakte wurden anschließend zur Vermessung mittels HPLC herangezogen.

Die Resultate zeigten einerseits, dass in beiden Kalluslinien weder Arbutin noch Bergenin in reproduzierbarem Ausmaß in den Suspensionsmedien zu finden waren. Weiters fanden sich auch keine detektierbaren Mengen an Arbutin in den Zellextrakten. Hinsichtlich der Auswirkung auf die Bergeninproduktion zeigte sich eine starke Dichotomie: während Linie BP 57 durch einige Behandlungen den zu erwartenden statistisch signifikanten Anstieg an Bergenin aufwies, kam es bei fast allen Behandlungen von Linie BP 179 zu einer signifikanten Reduktion des Bergeningehaltes. Mögliche Gründe für diese unerwarteten Ergebnisse wurden zu Ende der vorliegenden Arbeit diskutiert, doch bedarf es für eine zufriedenstellende Aufklärung weiterer gezielter Untersuchungen.

2. Introduction

2.1. *Bergenia pacumbis* (Buch.-Ham. ex D.Don) C.Y.Wu & J.T.Pan

Bergenia pacumbis is a perennial, herbaceous plant of the Saxifragaceae family which is mainly to be found in Asia, especially East, Central and South Asia (Zhang et al., 2011). It grows up to a height of approximately 20 cm and has basal, leathery leaves with an ovate blade that form a rosette; both surfaces are glabrous. The inflorescence is cymoid, approximately 7.5 cm long and has five sepals and petals; the petals can be of white to pink color. The rhizome is thick and scaly (Jintang & Soltis, 2001).

In Traditional Chinese Medicine, Ayurveda and traditional Nepalese medicine several members of the genus *Bergenia* (often without or with unclear distinction) are used for the treatment and cure of various ailments, including bladder and kidney diseases, heart and lung diseases, malaria and others. The drugs in use are in general derived from roots and rhizome, but leaf extracts are also described. This usage goes hand in hand with scientific findings, which show on the one hand that these (and other) properties can be attributed to different phenolic compounds and, on the other hand, that most of these compounds are to be found in the roots and rhizome (Pandey et al., 2020; Koul et al., 2020).

Two major compounds of *Bergenia* species, arbutin and bergenin, are connected with several different effects: for arbutin (hydroquinone β-D-glucopyranoside; fig. 1) the most well-known and exploited property is its antibiotic and diuretic activity, which is the basis for its use in the traditional treatment of urinary infections – the most common example is the application of bearberry extracts (Afshar et al., 2018; Moore et al., 2019). The use of arbutin-containing plant extracts for such uncomplicated infects could therefore be seen as a good alternative to the use of conventional antibiotics (Afshar et al., 2018). On the other hand there is evidence that shows no significant benefit in the use of such extracts (Moore et al., 2019). Another potential problem in its use (specifically: of hydroquinone) lies especially in the repeated or long-term application which has been supposed to increase different health risks because of hepato- and nephrotoxicity as well as carcinogenity of the compound. More recent studies seem however to either disprove or attenuate these assumptions (Jurica et al., 2018; de Arriba et al., 2013), there are even indications that arbutin might be an effective agent against bladder cancer (Li et al., 2011a).

Another field of use is the cosmetic industry or skincare sector, where the application of arbutin is aimed at depigmentation (i.e. treatment of hyperpigmentation; Maeda &

Fukada, 1996; Zaid & Al Ramahi, 2019). Even though hydrochinon-containing cosmetics have been banned in 2001 in EU countries, cosmetic plant products naturally containing arbutin and/or hydrochinon were not affected by this ruling and successive studies on (systemic) health risks also consider these applications as safe (O'Donoghue, 2006; SCCS & Degen, 2016).

More recently it has also been shown that arbutin might possess the ability to positively interfere with the development of neurodegenerative diseases (more specifically: Morbus Alzheimer) by impairing oxidative damages – most likely due to its antioxidant/radical scavenging properties (Dastan et al., 2019).

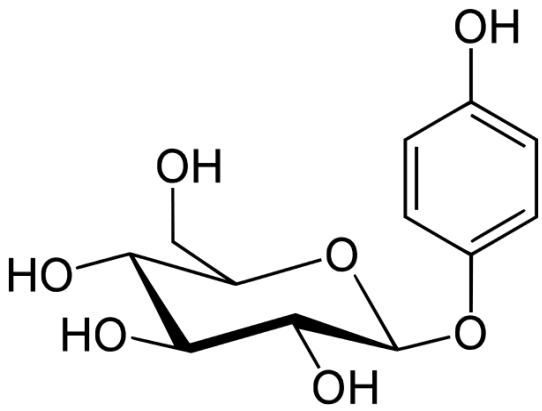

Figure 1. Chemical structure of arbutin. Image taken from: https://de.wikipedia.org/wiki/Arbutin#/media/Datei:Arbutin_structure.svg.

Bergenin (fig. 2) on the other hand has been associated with a variety of different effects, among which are antiulcer, (potential) anti-HIV (Prinsloo et al., 2018), neuroprotective, antiinflammatory and immunomodulatory activities (Qi et al., 2018; these and further examples see Zhang et al., 2011 referring to Nazir et al., 2011). Some of these are caused by very interesting molecular modes of actions: for example, the neuroprotective property is not only linked to the antioxidative effects, which bergenin shares with arbutin, but also a more specific inhibition of the acetylcholinesterase and other important targets (Pandey et al., 2020), similar to standard anti-Alzheimer medications like donepezil and galanthamine (Barai et al., 2019). Its antiulcer activity is furthermore connected to the inhibition of *Helicobacter pylori*, most likely due to a specific urease-inhibition; this enzyme plays a key role in the bacterium's ability to survive in the acidic environment of the stomach (Ali et al., 2020). Lastly, a rather recent study linked two important diseases, diabetes and osteoporosis, by showing how bergenin was able to reverse some of the mechanisms caused by methylglyoxal, a precursor of advanced glycation end products, and thereby decreasing

the elevated osteoclast activity (Suh et al., 2019). The study also points out the inhibition of specific methylglyoxal-activated signal molecules and/or genes, which leads to the question whether bergenin might also interfere with other methylglyoxal-linked changes (especially vascular) found in diabetes patients – a certainly interesting topic, which needs further elucidation.

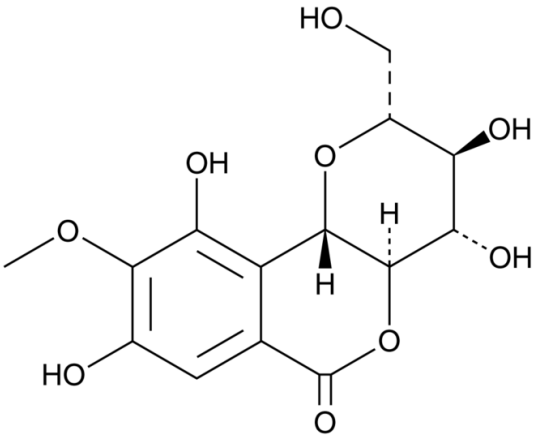

Figure 2. Chemical structure of bergenin. Image taken from: https://www.biomol.com/de/produkte/chemikalien/biochemikalien/bergenin-cay26406-250.

2.2. Plant cell and tissue culture

Plant cell and tissue culture (PCTC) can be defined as the aseptic cultivation, maintenance and propagation under *in vitro* conditions of plant cells, tissues, organs and organisms (Lange, 2018). It can be used for the micropropagation of plants on a large scale for agricultural purposes, in the cosmetic and ornamental industry, as well as for the preservation of endangered species (Mehrotra et al., 2007). Furthermore, the propagated plants themselves (*in toto* or specific organs), or callus and cell suspension cultures, can be used for the production of secondary metabolites (Siahsar et al., 2011; Weathers et al., 2010; Georgiev et al., 2009). The usage of plant tissue cultures for the production of secondary metabolites is mainly chosen when the plant or the metabolites is/are scarce under natural conditions. Furthermore the following advantages are (among others) often listed when comparing PCTC to field or greenhouse cultures (Cusido et al., 2014; Karuppusamy, 2009; Mehrotra et al., 2007; Debnath et al., 2006): the growth and harvesting is independent from local circumstances (be it economical or social problems in the regarding area or environmental challenges, like pollution, contamination of the soil but also use of pesticides, herbicides and the like) and therefore also very reliable; the growth conditions (light, temperature, humidity etc.) are in general easily controllable; the produced

plants are overall genetically homogenous and can be produced over a short time in a great amount; the plants are in general thought to be axenic (i.e. free from contaminations), and virus-contaminated plants (from existing field cultures) can be treated to be virus-free again; the phytochemicals produced in PCTC are better accessible (i.e. the isolation is quicker) than in field cultures; the amount of these secondary metabolites produced in PCTC is often also comparable to *ex vitro* plants and moreover it is generally thought to be upscaleable.

There are to date many interesting phytochemicals (and biopharmaceuticals) reported to be produced in PCTC (Table 1), however only few of them on an industrial level.

Table 1. Examples of substances produced in PCTC (Steingroewer et al., 2013; Yue et al., 2016; Ochoa-Villarreal et al., 2016; Santos et al., 2016; see these sources for further examples).

Plant (Species)	Product	Culture
Artemisia annua L.	Artemisinin	Cell suspension culture
Atropa belladonna L.	Atropine	Hairy root culture
Daucus carota L.	Taliglucerase alfa	Cell suspension culture
Hypericum hirsutum L., *Hypericum maculatum* CRANTZ	Hypericin, pseudohypericin, hyperforin	Shoot culture
Leucojum aestivum L.	Galanthamine	Shoot culture
Medicago truncatula GAERTN.	EPO	Cell suspension culture
Panax ginseng L.	Ginsenosides	Adventitious root culture
Papaver somniferum L.	Codeine	Cell suspension culture
Podophyllum peltatum L.	Podophyllotoxin	Adventitious root culture
Taxus spp.	Paclitaxel	Cell suspension culture

The main criterium, apart from the actual feasibility to produce a substance on a large-scale level using plant cell cultures, is the economic viewpoint (whether the cost-value ratio is beneficial). This however is (to date) only the case for paclitaxel (and derivatives), the most famous example for a substance produced in PCTC and for the potential opportunities that PCTC offers. This anti-cancer agent was originally isolated from the bark of the pacific yew, *Taxus brevifolia* NUTT. However, the destructive character of this method, combined

with low yields, resulted in a semi-synthetic approach, in which taxol precursors – baccatin III and 10-deacetyl baccatin III – were extracted from needles of the European yew, *Taxus baccata* L., and are now produced in cell cultures of *Taxus* spp. (Cusido et al., 2014; Kreis, 2019). Future approaches (due to the comparably slow growth of plant cells) might include bacteria and/or fungal cultures (see chapter 2.3.). Another example for a compound which has for a long time been produced in PCTC as well, but is now again extracted from *ex vitro* plants (since there has been shown no financial benefit) is the antibiotic agent shikonin, found in the roots of *Lithospermum erythrorhizon* SIEBOLD & ZUCC (Yakazi, 2017; Lange, 2018; Kreis, 2019).

The use of PCTC in the food and cosmetic industry on the opposite is extensive, with the main focus on polyphenolic elements, like flavonoids and anthocyanines, which can serve as food additives, supplements and coloring agents (Eibl et al., 2018; Kreis, 2019; Belwal et al., 2020).

Apart from pharmaceutically used small molecules there is also the possibility to produce transgenic proteins in PCTC, however the only product to date commercially produced in cell suspension cultures and not in *toto* plants (Santos et al., 2016; Edgue et al., 2017) is taliglucerase alfa, a recombinant glucocerebrosidase used for the treatment of Morbus Gaucher. Nevertheless, the possible industrial-level production of biopharmaceuticals in PCTC is given for further substances, like alidornase alfa (rhDNase-I, promising results were shown in phase II clinical trial for the treatment of cystic fibrosis) and pegunigalsidase alfa (recombinant α-galactosidase A, currently in phase III clinical trial for the treatment of Fabry's disease) (adapted from Xu & Zhang, 2014; http://protalix.com/pipeline/). Further compounds include different antibodies, vaccines and cytokines (Xu & Zhang, 2014; Hellwig et al., 2004) – however, it is uncertain whether methods for sustainable upscaling can or will be developed.

2.2.1. Callus culture

Beside the usage of *in vitro* plants (i.e. more or less complete organisms), callus is often the preferred alternative. It can be the result of a natural process following certain stress situations the plant is exposed to (like wounding, infections etc.), or it can be willfully induced on a suitable nutrient medium using explants from existing *in vitro* plant cultures, or surface-sterilized explants from intact plants, respectively (Georgiev et al., 2009; Fehér,

2019). In most cases a high auxin content is sufficient to cause the formation of callus, in some cases an addition of small amounts of cytokinins can be useful (George et al., 2008).

Callus itself is often defined as "(mass of) de-/undifferentiated cells"; even though this terminology has been criticized and (likely) more correct alternatives like "transdifferentiated" have been proposed (Fehér, 2019). Even though callus cultures show benefits like easy handling, comparably low requirements in regards to the medium and the culture conditions (growth in darkness), their main purpose is either the multiplication of plant material – since it is possible to regrow complete organisms out of (almost) every cell, due to totipotency (Sugimoto et al., 2011; Fehér, 2019) – or they are an intermediate step in the establishment of suspension cultures (Hall, 1991), which can then be used to produce the secondary metabolites of interest.

2.2.2. Suspension culture

Since a semi-solid callus medium is generally optimized for the growth of a specific callus line, the only difference to a liquid medium for suspension culture is the absence of the gelling agent. However, since the acquisition of secondary metabolites is usually the main goal, it is sought to achieve single cell (or at least: small aggregates) cultures, as the exposition to the medium is more complete and at least in some cases it is known that secondary metabolite formation correlates negatively with the aggregate size, or is at least rather associated with smaller aggregates (Patil et al., 2013; Satdive et al., 2015). This, on the other hand, requires a specific structural composition of the callus (sc. friable and preferably soft). If this property cannot be obtained (and maintained) by altering medium composition or via agitation of the vessel or medium alone, forced separation (e.g. by sieving) of the smaller and larger aggregates can be applied, as well as mechanical 'stress' (see chapter 4.5.) or ultimately the addition of pectinase (Mustafa et al., 2011; Lee et al., 2004). The cultures are then kept either on specific shakers (for small-scale approaches), or the medium within the bioreactor itself (instead of the vessel) is moved via stirring or airflow (Georgiev et al., 2009; Yue et al., 2016).

Since one of the main goals of these *in vitro* cultures is the production of secondary metabolites, several approaches are possible to increase product yield. Generally, the establishment of a growth curve is essential, since research has shown that the secondary metabolite production only starts in approximately the last third of the linear growth and

reaches its height at the stationary phase, which would therefore be the ideal timepoint for product recovery (Mustafa et al., 2011). In many cases a major disadvantage of cell suspension cultures is the low production of metabolites (Lorence et al., 2004; Shilpa et al., 2010; Wilson et al., 2018) Parameters that can influence the yield (are the use of specific (high-yield) cell lines (Murthy et al., 2014; Malik et al., 2016; Yue et al., 2016), optimization of media composition (Ochoa-Villarreal et al. 2016; Fidemann et al., 2018; Yue et al., 2016), addition of precursors (Karuppusamy, 2009; Vijaya Sree et al., 2010; Yue et al., 2016), metabolic-engineering (Yue et al., 2016; Karuppusamy, 2009; Fischer et al., 2015), elicitation (Yue et al., 2016; Cusido et al., 2014, Ochoa-Villarreal et al. 2016) as well as co-cultures (Weathers et al., 2010; Goers et al., 2014). Elicitation can be described as the reaction of plant cells towards exposure to abiotic and/or biotic stimuli (fig. 3) – abiotic stimuli include radiation, temperature, heavy-metals and the like; biotic stimuli on the other hand are organic compounds derived from living organisms like bacteria, fungi etc. (Karuppusamy, 2009; Cusido et al., 2014; Chong et al., 2005).

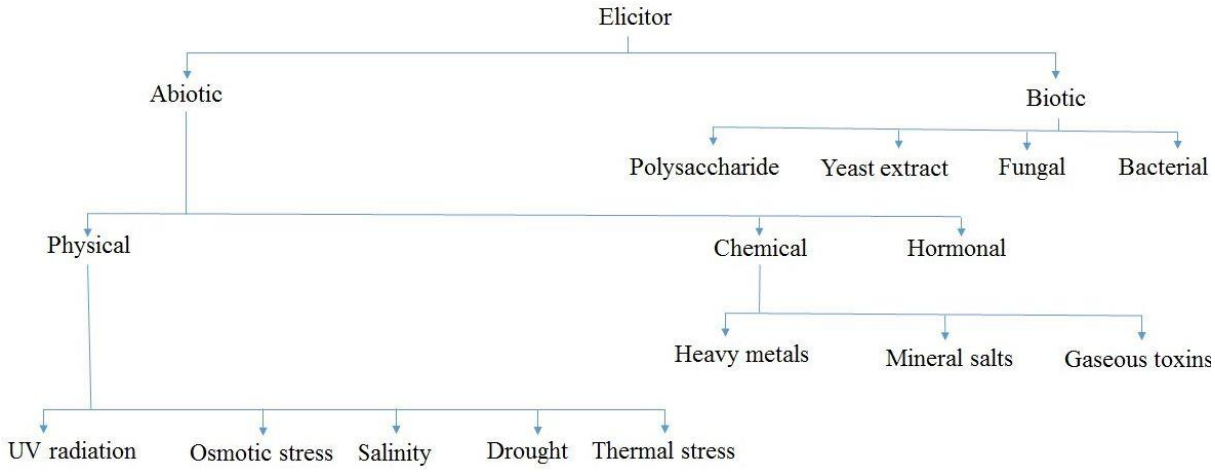

Figure 3. Mode of classification and different examples of elicitors (Naik & Al-Khayri, 2016).

As this process portrays the natural reaction towards pathogens or environmental stresses, plant signal molecules involved in stress pathways (for example methyl jasmonate or salicylic acid) have been shown to increase the production of secondary metabolites on their own when added (Giri & Zaheer, 2016; Frankfater et al., 2009). Co-cultures on the other hand can include both mixed cultures of different plant species, as well as plants and (in general) microorganisms (Weathers et al., 2010; Goers et al., 2014). Whether the main mechanism responsible for the increased production in this case is in fact the cell-cell-contact that mimics natural conditions, an actual defense mechanism (even though this would contradict

our general understanding of endophytes – see chapter 2.3.) or a form of support by the microorganism against future exposure towards actual pathogens, needs further elucidation.

2.3. Endophytes

The term *endophyte* was first used by De Bary (1866) and originally, as well as during the following period, simply used for microorganisms (primarily fungi) which inhabited a plant (hence the name, derived from greek *ἔνδον* 'inside' and *φυτόν* 'plant' – Kytzler et al., 2014) (Wilson, 1995). A more recent definition given by Wilson (1995) – "endophytes are fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease" – comes closer to our current understanding, even though the broad mass of research emphazises their beneficial (instead of only non-harmful) impact on plants. Furthermore it is certainly noteworthy (Wilson purposefully made this exclusion), that not only bacteria and fungi are known to be endophytes; research regarding endophytic algae, viruses etc. is however comparably underrepresented.

The following examples are only a small fraction of the findings made to date regarding beneficial effects endophytes can have on their plant host (Yan et al., 2019; Kandel et al., 2017; Khare et al., 2018): they are known to e.g. produce plant growth hormones, like *Bacillus subtilis* (Malfanova et al., 2011) and *Metarhizium robertsii* (Liao et al., 2017), help the host against abiotic and biotic stressors, like *Aspergillus* and *Penicillium* spp. against *Fusarium* sp. (Yan et al., 2019), *Pseudomonas* spp. against *Verticillium dahlia* (Prieto et al., 2011), *Bacillus subtilis* (Yan et al., 2019) as well as *Neotyphodium* sp. (Gundel et al., 2010) against salinity stress. They provide nutrients, e.g. N-fixation by *Gluconacetobacter diazotrophicus* (Eskin et al., 2014) and P-acquisition by *Bacillus* spp. (de Abreu et al., 2017) and stimulate the production of plant-specific secondary metabolites or even produce them themselves (examples see below; Jia et al., 2016; De Meyer et al., 2019; Taghinasab & Jabaji, 2020).

It is thought, that every plant contains at least one species of endophyte. The origin of this symbiosis is however uncertain – whether they were niche-colonizers (Kandel et al., 2017; Liu et al., 2017), individuals that showed low pathogenity and/or a suppressed immune response while infecting the host (De Meyer et al., 2019), or if their cohabitation is simply the result of a balanced antagonism (Schulz et al., 1999). The acquisition of

endophytes can occur vertically (from an adult individual to its offspring), horizontally (from one adult individual to another or from the surrounding environment) or via a mixed-mode (Bright & Bulgheresi, 2010; Frank et al., 2017).

It has been observed that plant hosts select certain environmentally occurring microorganisms, which leads to a decrease of biodiversity and abundance of endophytes compared to rhizosphere microorganisms (Luo et al., 2019; Nuccio et al., 2016; Wagner et al., 2016). The result of this phenomenon is the so-called “core microbiome”, a certain composition of different genera, which is, however, consistent and similar within and even among various plant species and can be conserved throughout the generations (Walitang et al., 2019; Rodríguez et al., 2018; Truyens et al., 2014). The reason for this selection lies in most cases in a beneficial effect gained from endophytes. This can be resistance towards drought stress (Mitter et al., 2017; Marasco et al., 2012), gain in growth hormones (Mitter et al., 2017; Kandel et al., 2017) or sometimes even the ability to germinate successfully (Truyens et al., 2013; Shahzad et al., 2018) or to increase the germination rates (Gagic et al., 2018; David et al., 2019; Yan et al., 2019). The mechanisms involved in this selection process can be seen as a combination of simple physical characteristics (e.g. high osmotic pressure in seeds; Truyens et al., 2014), general adverse immunological reactions of the host (Compant et al., 2010; Truyens et al., 2013) in combination with a suppressed immunological response for certain species by the host (Irieda et al., 2019; De Meyer et al., 2019) on the one hand, and antimicrobial activities of already established endophytes (Raaijmakers et al., 2008; Compant et al., 2010; Pandey et al., 2019) on the other hand.

Interestingly, the reverse effect of active recruitment of certain beneficial endophytes, has also been shown (Marasco et al., 2012; Kandel et al., 2017; Urbina et al., 2018). The ‘tools’ used in this selection and/or recruitment process are in parts elucidated: it is on the one hand the recognition of possible intruders of the endosphere via different pathogen/microbe-associated molecular patterns (PAMPs/MAMPs). Among these are surface molecules (like lipopolysaccharides, LPS, and exopolysaccharides, EPS), flagella (or more specifically: flagellin), bacterial secretion systems (especially type III) and others (Bulgarelli et al., 2013; Kandel et al., 2017; Pinto-Carbó et al., 2018). The recognition of fungal intruders seems to happen via the fungal cell wall, specific proteins (like cell wall degrading enzymes and toxins) as well as (toxic) secondary metabolites; furthermore, the often tissue-disruptive invasion (by using appressoria) is also most likely involved (van der

Does & Rep, 2017; Gow et al., 2017). Interestingly, these and other factors have been shown to be (at least partly) essential for colonization (Compant et al., 2010; van der Does & Rep, 2017). Therefore these microorganisms (beneficial as well as pathogenic) tend to mask certain elements of this recognition pattern – e.g. shedding their flagella during entry (Bent & Mackey, 2007) or modifying their cell wall (Gow et al., 2017). Another mechanism is the use of quorum sensing (QS) (González & Venturi, 2013; Kandel et al., 2017; Alagarasan et al., 2017), which is necessary on the one hand for bacteria to establish communities via environment-recognition (Hartmann et al., 2014). On the other hand, QS-molecules (N-acyl homoserine lactones, or: AHLs) have been shown to affect plant hormone levels (auxins and cytokinins, as well as their proportion) and thus create beneficial effects for the host (González & Venturi, 2013; Hartmann et al., 2014; Kandel et al., 2017; Alagarasan et al., 2017). Additionally a LuxR-related group (which normally responds to presence of AHLs) has been shown, in plant-associated bacteria and bacterial endophytes, to create a response to plant molecules instead (González & Venturi, 2013; Schaefer et al., 2013; Kandel et al., 2017). Another selection mechanism is the ability of potential endophytes to cope with plant defense molecules, like jasmonic acid (JA), salicylic acid (SA) and reactive oxygen species (ROS) (Compant et al., 2010; Kandel et al., 2017; Meneses et al., 2017). The actual recruitment or attraction has furthermore been shown to function via root exudates – i.e. molecules that are actively excreted from the plant into the soil (Bulgarelli et al., 2013; Kandel et al., 2017; Hartman & Tringe, 2019). Among these are sugars, vitamins, amino acids, phenolic compounds and others – often released as a response to (a)biotic stressors (Bulgarelli et al., 2013; Kandel et al., 2017; Hartman & Tringe, 2019). The recruitment has been well documented for the roots, but has also been shown to be present in the spermosphere (Nelson, 2004; Frank et al., 2017; Vujanovic et al., 2019) and the phyllosphere (Bulgarelli et al., 2013; Mina et al., 2020).

Not only plants themselves benefit from endophytes, but they can provide opportunities for the agricultural use of plants, such as wheat, maize, rice and more (Lodewyckx et al., 2002; Lugtenberg et al., 2016; Omomowo & Babalola, 2019), as well as medicinal plants. Furthermore, they have been shown to produce substances of interest together with medicinal plants (see the following chapter) or by themselves, e.g. paclitaxel (Zhou et al., 2010; Kusari et al., 2014), camptothecin (Kusari et al., 2009; Uzma et al., 2018), diosgenin (Li et al., 2011b; Xiang et al., 2018) and many others (Zhao et al., 2010; Brader et

al., 2014; Mohana Kumara, et al., 2014; Jia et al., 2016). The ability to produce (some of) these (and other) substances themselves could make them an alternative to slowly growing or problematic PCTCs (see e.g. Kusari & Spiteller, 2011; Ludwig-Müller, 2015; Xu et al., 2018).

2.4. Elicitation through endophytes

Like mentioned before, elicitation is often used in PCTC to increase the amount of secondary metabolites produced via an abiotic or biotic stimulus. The most common stimuli are yeast extracts, SA/JA and different anorganic compounds (Shilpa et al., 2010; Savitha et al., 2006; Giri & Zaheer, 2016). Only in the last couple of years the trend towards elicitation via plant-native endophytes has increasingly been investigated and shown promising results.

The underlying mechanism in the actual 'adverse' reaction of the plants against the beneficial microorganisms can be seen as priming for future defense reactions (Conrath et al., 2015; Liu et al., 2017) – like it has been shown for induced systemic resistance (ISR) caused by beneficial rhizospheric microbes (Conrath et al., 2015; Liu et al., 2017). Furthermore, it seems as if this rather unspecific response towards potentially harmful stimuli might at least be involved, if not even the (or: one of the) main reason(s) for the production of different secondary metabolites. A rather detailed summary of two specific pathways, the catharanthine biosynthesis in *Catharanthus roseus* and β-thujaplicin biosynthesis in *Cupressus lusitani*, given by Zhai et al. (2017), depicts the cascade – in both cases caused by endophytic fungal elicitors – from unspecific stress substances, like H_2O_2, over signaling molecules, like JA and SA, until the production of these interesting secondary metabolites.

A similar mechanism has been described by Zhou et al. (2016) for *Atractylodes lancea in vitro* plantlets inoculated with its endophytic bacterium *Pseudomonas fluorescens*. Here the authors suggested that the reactive increase of H_2O_2 led to an increase in gibberellic acid and ethylene; gibberellic acid, together with increased abscisic acid (ABA), finally led to the activation of key enzymes in the production of sesquiterpenes, which ultimately caused the increase of volatile oils (Zhou et al., 2016). In a later study of the same model (Zhou et al., 2018), the authors showed that an increase in indole acetic acid, caused by the inoculation with the endophyte (specifically: bacterial protein and polysaccharides), resulted in an increase of oxygen-free sesquiterpenoids. Similar observations have been made by Wang et al. (2015) – here increased levels of SA and ABA have been connected with the increase of

volatile oils in *Atractylodes lancea* plantlets elicited with *Acinetobacter* sp. – and Hao et al. (2010), who showed an increase of ABA in *Ginkgo biloba* suspension cultures when elicited with mycelium of the endophytic fungus *Sphaeropsis* sp., which was linked to the increase in flavonoid production in these cultures.

Another interesting example is the secondary metabolite production in hairy root cultures of *Salvia miltiorrhiza*. On the one hand it has been shown that both treatment with bacterial (Yan et al., 2014; here as a co-culture) as well as fungal endophytes (Ming et al., 2013; Zhai et al., 2018) could increase the amounts of tanshinones. On the other hand, the bacterial endophytes caused a decrease in the production of rosmarinic acid and salvianolic acid B (either caused by the degradation of these compounds by the endophytes or by the inhibition of the producing enzymes) (Yan et al., 2014), whereas with fungal elicitors the production was either effected non-significantly or only moderately (Ming et al., 2013) or even showed an increase in these metabolites (Zhai et al., 2018 – although these data are not exactly comparable, as the authors focused on long term changes). Furthermore, the bacterial elicitors showed an inhibition of the hairy root growth (Yan et al., 2014), whereas the fungal elicitors showed an increased growth of either the hairy root (Ming et al., 2013) or the seedlings (Zhai et al., 2018). The involvement of defense reactions against endophytic fungi has also been shown by Jiang et al. (2019), who performed a transcriptome analysis of *Salvia miltiorrhiza* co-cultivated with its endophytic fungi. A causal link between the initial expressionincrease of defense genes and the also increased expression of tanshinone biosynthesis genes was however not investigated here.

Such results further indicate, that the adverse reactions are not a response towards 'unspecific' biotic elicitors (since many elicitation experiments are performed with autoclaved cells, mycelia or cell-specific compounds like cell-wall molecules or proteins – Wang et al., 2012; Zhong et al., 2016; Zhou et al., 2018) but are also received when the plant is treated with living organisms – like in co-cultures. The benefit of these culture systems has for example been shown in case of the increased paclitaxel production by *Taxus chinensis* var. *mairei* suspension cultures when co-cultured with its fungus *Fusarium mairei* (Li et al., 2009), the increased podophyllotoxin and 6-methoxypodophyllotoxin production in *Linum album* suspension cultures when (separately) co-cultured with *Piriformospora indica* and *Sebacina vermifera* (Baldi et al., 2010; the authors also showed in this study the beneficial effect of elicitation with the fungal culture filtrates) and the increased production of emodin,

resveratol and other metabolites in *Rumex gmelini* seedlings when co-cultured with *Aspergillus* sp., *Fusarium* sp., and *Ramularia* sp. (Ding et al., 2018). Furthermore it has been shown, that the co-culture concept does not only increase the amount of secondary metabolites produced by plants, but reversly also that endophytic organisms exhibit an increased biosynthesis of the compounds when co-cultivated with their native host plant (Li et al., 2012; Salehi et al., 2018; Wang et al., 2019).

3. Aim of this work

The aim of this work was to investigate possible influences of endophytes, isolated from *Bergenia pacumbis*, on the production of phenolic secondary metabolites (specifically bergenin and arbutin) in cell suspension cultures of this plant.

The following tasks were therefore part of this thesis:

- Establishment (and optimization) of cell suspension cultures

Previously callus established on semi-solid medium was inoculated into a variety of liquid media in order to grow fine (optimally: single) cell suspension cultures.

- Growth curve measurements

A number of cell lines of these suspension cultures have been selected to measure their growth and thereby determine a suitable elicitation timepoint.

- **Identification and classification of bacterial endophytes from *Bergenia pacumbis***

A selected number of endophytes, isolated from individuals of this plant, were identified using 16S rDNA sequencing, and phylogenetically classified.

- Elicitation of cell suspension cultures

Autoclaved cells as well as sterile filtrated bacterial medium of selected endophytes have been used to elicit *B. pacumbis* suspension cultures.

- Extraction of secondary metabolites and their quantification via HPLC

Extracts of lyophilized callus cells and medium filtrate were examined for their content of secondary metabolites of interest using a modified standard HPLC method.

4. Material & Methods

4.1. Plant & callus material

The original stock plant of *Bergenia pacumbis* was collected in Phulchoki, Nepal, and brought to Vienna in June 1993. It has been kept in the greenhouse of the Department of Pharmacognosy (University of Vienna), and clonally propagated offshoots are cultivated in the medicinal plants garden ibidem (Höninger, 2020; Malla, 1999).

The callus lines for this thesis were established by Höninger during a previous diploma project by harvesting, surface-sterilizing and cultivating different explants from *Bergenia pacumbis* leaves within the period of October to December 2017 (Höninger, 2020). The ones used during this project specifically were petiole- (BP 19 & BP 179), midrib- (BP 57 & BP 121) and lamina- (BP 131) derived lines, all of which grown on medium 2 (Höninger, personal communication).

4.2. Culture media

The callus cells were grown on semi-solid and liquid variations of the MS basal medium (Murashige & Skoog, 1962). The media were prepared in a large Erlenmeyer flask by first adding successively the required amounts of stock solutions (fig. 4) – which were used for macro-, microelements, vitamins and plant growth regulators. Of the latter NAA (Sigma-Aldrich, N0640), BAP (Sigma-Aldrich, B3408), and, for testing purpose only, 2,4-D (Sigma-Aldrich, D7299) were used during this thesis. Then the mixture was filled up with distilled water to almost the desired volume and the prescribed quantities of solids (fig. 4) – sucrose (food product quality), myo-inositol (MERCK, 4125) and, for semi-solid medium only, Gelrite® (Roth, 0039.1) – were added. Finally the flask was filled up with distilled water and the pH was adjusted to 5.7 ± 0.1 using 1 mM NaOH.

10 ml/l KNO_3 (100x)	5 ml/l Vitamine-solution (200x)
10 ml/l NH_4NO_3 (100x)	1 ml/l Microelements (1000x)
10 ml/l $CaCl_2$ (100x)	30 g/l Sucrose
10 ml/l KH_2PO_4 (100x)	100 mg/l Myo-Inositol
10 ml/l $MgSO_4$ (100x)	3 g/l Gelrite®
10 ml/l $FeSO_4$ (100x)	
10 ml/l NAA (1 mM)	
10 ml/l BAP (1 mM)	
10 ml/l 2,4-D (1mM)	

Figure 4. Used amounts of stock solutions and solids for the preparation of MS media. The parentheses contain either x-fold or molar concentration of the used solution. Standard medium 2 did not contain 2,4-D; for the tested derivatives of medium 2 the added amounts of plant growth regulators differed according to the desired concentration (see chapter 4.4.); media for suspension cultures did not contain Gelrite®.

40 ml of the prepared suspension (for semi-solid media) were filled into HiPP® babyfoodjars (h: 6.5 cm, d: 5.5 cm) and closed with Magenta™ B-caps (Sigma-Aldrich, B8648). For the liquid media, initially 100 ml Erlenmeyer flasks were filled with 20 ml of the solution, later on 250 ml flasks were filled with 50 ml. They were closed with suitable cellulose plugs afterwards. All media were finally autoclaved for 20 minutes at 121 °C and 1 bar. The media were then stored at 10° C until use.

Medium 2 was the only semi-solid medium used during the entire project, containing 10 µM NAA and 10 µM BAP (Höninger, 2020); for suspension media several concentrations of growth regulators had to be tested (see chapter 4.4.).

4.3. Culture conditions

All cultures were kept in the dark at 25 ± 1 °C; those in liquid media were incubated on a rotary shaker at 120 rpm. To avoid extensive evaporation of the liquid medium during the cultivation a piece of aluminum foil was used to cover the cellulose plugs. Callus on semi-solid medium was subcultured approximately every 4 weeks, suspension cultures in liquid media every 2 weeks.

4.4. Maintenance and gain of callus cultures

To gain enough material for experiments regarding the establishment of cell suspension cultures, the used five callus lines (see above chapter 4.1.) had to be propagated. This happened in an interval of approximately 4 weeks. The large cell aggregates were cut into smaller pieces, which were then placed into jars with fresh medium. After closing with Magenta™ B-caps, a small remaining gap between lid and glass was sealed with Parafilm® M (Witeg) to further prevent any form of (cross-) contamination. The vessels were then kept under the described conditions and further subcultured until enough material was available.

4.5. Establishment of cell suspension cultures

For the establishment of cell suspension cultures, callus from semi-solid medium was transferred into initially 100 ml Erlenmeyer flasks, containing 20 ml of liquid medium (approx. 2 g of callus), later on into 250 ml Erlenmeyer flask with 50 ml of liquid medium (approx. 3 g of callus). For further subculturing of the newly formed larger callus aggregates, they were separated from the medium (containing single cells and small cell aggregates) by using a tea sieve. Subsequently, they were chopped into smaller pieces and used as inoculum for fresh medium. The old medium on the other hand, with the single cells and small aggregates, was collected in an empty sterile Erlenmeyer flask. After approximately 10 minutes of letting the cells sediment, most of the supernatant medium was pipetted off and discarded; the remaining suspension was inoculated into 20 or 50 ml of fresh medium. Depending on the amount of suspension obtained through this procedure (and the volume of the new medium), 5 to 20 ml served as inoculum for the new cultures.

Further approaches to achieve single-cell suspension cultures consisted of:

.) Testing of various derivatives of standard medium 2, differing in the amount and composition of plant growth regulators (see table 2).

Table 2. Plant growth regulator contents for the different tested media.

Medium	NAA (µM)	BAP (µM)	2,4-D (µM)
2	10	10	-
2a	10	5	-
2b	10	2.5	-
2c	5	10	-
2d	5	5	-
2e	10	10	5
2f	10	10	10
2g	5	5	5
2h	5	5	10

.) Addition of pectinase (in order to dissolve the the middle lamella), using a stock solution of 250 mg pectinase (namely pectinase from *Rhizopus* sp.; Sigma-Aldrich, P2401) in 50 ml distilled water. This solution was then sterile filtrated and aliquots of 200 µl were added to 20 ml medium (or 500 µl to 50 ml, respectively), in order to achieve a final concentration of 0.005% (Lee et al., 2004; Mustafa et al., 2011; other than described by Lee et al., pectinase was only added once, at the day of inoculation). The pectinase stock solution was stored at -20 °C for later use.

.) An attempt adopted from Williams et al. (1988) consisted of blending of callus aggregates (from semi-solid medium) with liquid medium (only medium 2 was used) at 15,600 rpm for four seconds (blender model: Waring 32BL79).

.) The use of glass beads as mechanical treatment has also been tried by adding glass beads of 2 or 3 mm in diameter (3 or 4 g per 50 ml medium, respectively). As this method has been derived from the treatment of bacteria cultures the speed of the rotary shaker had to be adjusted as well – initially to 150, later to 200 rpm.

All of these other attempts included the separating procedure using the tea sieve as well.

Especially in the beginning of the different methods viability tests have been performed. For the evaluation of viability fluorescein diacetate (FDA; Sigma-Aldrich, F7378) has been used (following Widholm, 1972). First a stock solution has been prepared, containing 5 mg FDA in 1 ml acetone. Of this stock solution 10 µl have been added into 1 ml

of distilled water. From the suspension culture 500 µl were taken and put into an Eppendorf tube as well. Both have been stored on ice before the experiment was performed. One drop of the cell suspension tube (containing mostly the sedimented single cells) was put on a specimen slide with a Pasteur pipette and one drop of the FDA-solution was added and stirred. After 3 minutes of incubation the slide was put under a fluorescence invert microscope and the amount of cells per sighting field (under bright-field illumination) counted and compared to the amount of cells fluorescing under a UV-filter (excitation wavelength 450-490 nm).

4.6. Growth curves of cell suspension cultures

For the determination of a proper elicitation timepoint, a growth curve of the cell lines had to be established. The inoculation of 50 ml liquid medium with 4 ± 0.16 g of small callus aggregates occurred as described above. The method for growth measurement was adopted from Blom et al. (1992). Five Erlenmeyer flasks per callus line have been used and measured with an apparatus similar to the one described by Blom et al., which allowed the cells to sediment for 5 minutes (initially) or 3 minutes respectively (since it has been determined during the first conducted experiment, that after 3 minutes there was no change in the measured height detectable) at a constant angle of 60°, and the height of the sediment to be determined by an attached scale. To allow a correlation of the measured height and cell volume after sedimentation a straight calibration line had to be established using Microsoft Excel 2013 (Microsoft Corp.). The measurements for the growth curve took place every other day (the initial measurement occurred on the day of inoculation) until the start of the death phase had been reached.

4.7. Identification of endophytes

The endophytes used for identification (and elicitation) were originally isolated from *Bergenia pacumbis* by Dr. Martina Oberhofer and stocks thereof kept in a freezer at -80 °C. It is noteworthy, that the denomination of the endophyte samples, “BL” (see chapter 5.3.), represents a previous state of research, when the plant was classified as *Bergenia ligulata* – the current status is represented by the abbreviation “BP”, used for the callus cultures. With

an inoculating loop, small aliquots of the still semi-solid isolates were put into 13 ml vials with 2 ml of TSB medium and incubated in a shaker over night at 200 rpm and 28 °C to generate seed-cultures. These were then centrifuged at 4000 rpm for 10 minutes and the resulting bacteria pellets, after discarding the supernatant, were transferred into 1.5 or 2 ml Eppendorf tubes and stored at -20 °C. For the most part, these pellets had already been generated by Dr. Oberhofer and Mag. Höninger. If no bacteria pellet of a relevant strain was available in the -20 °C freezer, they were generated by myself.

4.7.1. DNA extraction & purification

After thawing, the samples were put into a microcentrifuge and centrifuged for 3 min at 13,000 rpm. Occurring supernatant was discarded. For the extraction the NucleoSpin® Microbial DNA kit (Macherey-Nagel, 740235) was used. The pellets were resuspended in 100 µl BE, elution buffer, and – depending on the available sample mass – an appropriate amount of the resulting suspension (in most cases 50 – 75 µl were sufficient) was put into a NucleoSpin® Bead Tube Type B. Then 40 µl of MG buffer and 10 µl of Proteinase K were added. The tubes were put on a tissue lyser (Precellys® 24 lysis & homogenization, Bertin Instruments) at 4000 rpm for 60 seconds. Another centrifugation step (30 s at 11,000 rpm) followed and 600 µl of MG buffer were added. The samples were vortexed (for at least 3 s) and again centrifuged (30 s at 11,000 rpm). In a next step, the supernatant from the bead tubes was put onto a NucleoSpin® Microbial DNA Column inside a collection tube. The tubes were centrifuged (30 s at 11,000 rpm), the flowthrough was discarded and the columns were put into new collection tubes. 500 µl of BW buffer was added and the tubes centrifuged (30 s at 11,000 rpm), the flowthrough was discarded and the columns were put back again into the collection tubes. This step was repeated with 500 µl of B5 buffer. Then the columns were again centrifuged empty (to dry the silica membrane). The flowthrough and collection tubes were discarded and the columns put into 1.5 ml autoclaved Eppendorf tubes. 100 µl of BE buffer was added and the tubes left to incubate for at least 1 minute. Finally the samples were put into the centrifuge and after centrifuging for 30 s at 11,000 rpm, the columns were discarded and the tubes put on ice before gel electrophoresis was performed.

4.7.2. Gel electrophoresis

For the gel electrophoresis, 1.6 g of agarose (Sigma-Aldrich, A9539) were dissolved in 200 ml 1x TBE buffer, heated in a microwave until the solution was clear and 5 µl / 100ml GelRed® (Biotium, 41002) were added to create a 0.8% (m/V) gel-precursor. This solution was stored at 60 °C to prevent premature polymerization and poured into chambers with a comb 20-30 minutes before the electrophoresis was performed. As a reference for the evaluation of the quantity of the sample, 1 µl of a 1 kB ladder (BioLabs New England, N0468S) was used, which was put into the first well of the gel. Into each of the remaining wells (leaving one blank next to the reference) a sample containing 1 µl of purple loading dye (BioLabs New England, B7024S), 8 µl of 1x TBE buffer and 1 µl of extracted DNA – these compounds were thoroughly mixed – was pipetted. The electrophoresis was performed using 100 V for 40 minutes. Afterwards, the gel was evaluated with the ChemiDoc™ Touch Imaging System (Bio-Rad). Successfully extracted genomic DNA was identified by showing bands at a length larger than 10 kB on the reference ladder.

4.7.3. PCR

Since the endophytes of interest for this thesis were bacteria, the 16S rDNA was used for further phylogenetic characterization. The previously extracted DNA material was used for amplifying the 16S rDNA via PCR using standard 27F and 1492R as forward and reverse primers. For the PCR, a mastermix was prepared, containing:

0.5 µl Taq-Polymerase (BioLabs New England, M0273S)

0.5 µl dNTP-mix (BioLabs New England, N0447L)

1 µl 27F-primer (10 µM)

1µl 1492R-primer (10 µM)

4 µl 10x Standard Taq reaction buffer (BioLabs New England, B9014S)

and sterile distilled water per sample; however, since the amount of used DNA-sample varied – depending on the quantity of extracted DNA – the portion of distilled water varied accordingly. The total volume used for the PCR was 40 µl (i.e. if 1 µl of sample was used, the according amount of water was 32 µl, if 6 µl of sample were used, the amount was 27 µl and so on). PCR tubes were filled with the respective amount of PCR mastermix, the extracted

DNA sample was added and the compounds mixed. The tubes were put into the Mastercycler Nexus X2 (Eppendorf); the conditions for the PCR were the following:

initial denaturation at 95 °C for 2 min

30 cycles of:

denaturation at 95 °C for 30 s

annealing at 56 °C for 30 s

elongation at 72 °C for 3 min

the final elongation was performed at 72 °C for 5 minutes and afterwards the samples were stored at 4 °C until removal from the Mastercycler.

The PCR product then underwent another gel electrophoresis to judge its quality and quantity. The preparation of the gel and the reference was equal to the aforementioned process. However, the preparation of the samples and the evaluation differed: here 20 µl of PCR product were mixed with 2 µl of loading dye and pipetted into the wells (since the complete product was used, two wells of the same sample were created). The electrophoresis was again performed at 100 V for 40 minutes. After putting the tray into the ChemiDoc™ Touch Imaging System the samples were exposed to UV light outside of the system, making the 16S rDNA products visible at around the same height as the 1.5 kB reference. These lanes were then cut out, put into Eppendorf tubes and stored at -20 °C for further preparation.

4.7.4. Purification of amplicons

The following purification process was performed using the Zymoclean™ Gel DNA recovery kit (Zymo Research, D4002). For the two cut-out gel slices 700 µl of ADB buffer were used, which was added into the tube. The tubes were put on a prepared heating block at 50 °C for 10 minutes (until no gel remnants were left). This solution was then transferred onto a Zymo-Spin™ Column in a collection tube and centrifuged for 45 s at 13,400 rpm (due to the amount of the solution, this step had to be performed twice); the flowthrough was discarded. Next, 200 µl of DNA Wash Buffer were added to the column and centrifuged for 30 s at 13,400 rpm; the flowthrough was discarded and the washing step repeated, according to the protocol. Lastly the column was placed into a 1.5 ml Eppendorf tube, 15µl of DNA Elution Buffer were added and – to improve the yield – let incubate for at least 2 minutes; afterwards it was centrifuged for 45 s at 13,400 rpm. The column was discarded

and the Eppendorf tube with the flowthrough put into the freezer until a gel electrophoresis (according to 4.7.2.; to evaluate the purification product – lanes should be visible at the height of 1.5 kB of the reference) was performed.

4.7.5 Sequencing & phylogenetic analysis

The purified products were then prepared for sequencing, using the Mix2Seq kit (Eurofins Genomics). Aliquots of the products (amounts depended on the results of the gel electrophoresis) were pipetted into microtubes, adding 2 µl of forward or reverese primer respectively and an adequate amount of distilled water to achieve a total volume of 20 µl (e.g. 2 µl sample + 2 µl primer + 16 µl H_2O). The tubes were closed with plastic lids and sent to eurofins for sequencing. The sequences were received via email and edited, aligned and combined using the program Sci Ed Central (version 1.3.0.0; Sci Ed Software LLC); the achieved assembly was submitted to the online databases RDP Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp), Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (https://www.ezbiocloud.net/) to obtain the most probable result.

The results were then phylogenetically classified using the software MEGA X (version 10.2.2.; Pennsylvania State University). For this, the endophyte sequences and (for reference) the sequences of the closest matching type strains (obtained from the Nucleotide database of NCBI - https://www.ncbi.nlm.nih.gov/nuccore) were added to the software, alignments were performed (by use of the ClustalW algorithm on standard setting) and the resulting alignment was trimmed at the 5'- and 3'-ends to receive a more or less homogenous block of 1314 bases length, which was checked for any mistakes or implausibilities. For maximum likelihood analysis, the best fitting model was evaluated (again on standard setting). The result with the lowest BIC value (sc. 15587.804) was chosen as the best fitting model. Maximum-likelihood analysis was then performed using the optimal model (Kimura 2-parameter model; -Ln = 7288.775) and gamma-distribution. Finally, the bootstrapping method (with 500 replicates) was applied to estimate probabilities of branches. The resulting tree and automatically generated information on the statistical methods and work-process were saved (Instructions kindly received by Dr. Oberhofer).

4.8. Elicitation

4.8.1. Microbial material

The elicitation of the *B. pacumbis* cell suspension cultures was carried out using five strains of the above mentioned endophytes, which were identified as described before. The final selection of the endophyte strains was based on the aspects of phylogenetic diversity and/or the known production of interesting metabolites by the organism.

4.8.2. Bacterial growth curves

The generation of seed cultures was conducted as decribed above (see chapter 4.7.), however this time both LB and TSB media were used. 10 µl from the resulting seed cultures were pipetted into 250 ml Duran® Erlenmeyer flasks (with DIN thread and PBT cap) with 50 ml of the corresponding medium and cultivated under the same conditions as described above until the end of this experiment. The first measurement of the optical density (OD), using a UV/VIS-spectroscope at a wavelength of 600 nm with the software Thermo Scientific™ INSIGHT™2 (Thermo Fisher Scientific Inc.), was performed 24 h after the inoculation with the seed-cultures, using 1 ml of the culture suspension; an aliquot from a flask of un-inoculated (equivalent) medium, which was kept under the same conditions, served as blank. If necessary (sc. in case that OD > 2) the measuring samples had to be diluted 1:10 with the corresponding medium to achieve exact results. Further two measurements per day (after 2 hours each) were conducted until the stationary phase or the beginning death phase of the growth curve was reached.

4.8.3. Elicitation experiment

The elicitation timepoint was set according to the established growth curve for the two *B. pacumbis* cell lines. As elicitors 200 mg of autoclaved bacterial cells and 5 ml of sterile filtrated (bacterial) medium, respectively, were chosen. To gain the bacteria cells, the cultures were autoclaved for 20 minutes at 121 °C and 1 bar after reaching the stationary phase of their growth. The content of the flasks was then pipetted into 50 ml Falcon™ tubes (which had been weighed beforehand) under sterile conditions and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the Falcon™ tubes weighed. The bacterial cell pellet was then resuspended in sterile distilled water so that 5 ml contained 200 mg of cells. To obtain the medium elicitor, the content of the Erlenmeyer flasks was

pipetted under sterile conditions into 50 ml Falcon™ tubes as well. The Falcon™ tubes were then centrifuged at 10,000 rpm for 10 minutes, so that bigger cell aggregates wouldn't immediately clog the sterile filter. The sterile filtration was then performed into another 50 ml Falcon™ tube. Both the suspension with autoclaved bacteria cells as well as the sterile filtrated medium were stored in a freezer at -20 °C until use.

For each bacterial strain and the respective medium, as well as for the media blank measurements and untreated cultures, three Erlenmeyer flasks of suspension cultures (containing 4 ± 0.1 g of callus each) were treated accordingly, and after 48 h of incubation under the described conditions the cultures were filtrated through a Büchner funnel (for 5 minutes). The filtrate was collected in a 100 ml round bottom flask, the callus cells (and filter paper) in a 50 ml Falcon™ tube (which had been weighed beforehand to determine the fresh weight) and both were stored in a -20 °C freezer until lyophilization.

4.9. Sample preparation

The following methods for metabolite extraction and preparation for the measurements, as well as the standard preparation were adopted from Höninger, who followed the elaborations of Boros et al. (cf. Höninger, 2020 and Boros et al., 2014). The Falcon™ tube containing the lyophilized callus of the respective treatment was first weighed (to determine the dry weight), then a part of the callus was pulverized and 30 mg thereof were put into a 2 ml Eppendorf tube. Subsequently, 500 µl of a 1:1 (V/V) methanol-water solution were added and the mixture sonicated for 30 minutes. The suspension was centrifuged for 10 minutes at 13,400 rpm and the resulting supernatant was pipetted into a 1.5 ml Eppendorf tube, which was then stored at 4 °C in the dark. The procedure for the lyophilized media in the 100 ml round bottom flasks was similar, however here, after scratching the lyophilisate out of the flasks and weighing it, 30 mg were chopped (as far as possible) with a spatula and put into the Eppendorf tube. The remaining material was put into a 2 ml Eppendorf tube as well and stored (together with the Falcon™ tubes) inside an exsiccator. Before the measurement was conducted, the extracts were centrifuged again for 10 minutes at 13,400 rpm, 100 µl were then pipetted into the HPLC vials and 10 µl injected for the analysis. As a standard solution a 1:1 (m/m) mixture of arbutin (TCI; A0522) and bergenin (TCI; B4349) (in different concentrations) with methanol as solvent was prepared. For a complete dissolution, the volumetric flask with the components was put for 10 minutes

into an ultrasonic bath. 100 µl of the solution were pipetted into the HPLC vial and 5 µl were injected.

4.10. High Performance Liquid Chromatography (HPLC)

Analysis of arbutin and bergenin in the elicitated cell suspension cultures was conducted by High Performance Liquid Chromatography (HPLC).

4.10.1. Device & Conditions

The components (cf. Höninger, 2020) of the HPLC device (Shimadzu Corp.) were a LC-20AT pump, a DGU-20A5R degasser, a CTO-20AC column oven, a SPD-M20A photodiode array detector and a CBM-20A controller. For the injection of the samples a SIL-20AC HT autosampler was used. The column model was a Luna® C18(2) 100 Å 5 µm (Phenomenex Ltd.), with a length of 250 mm and a diameter of 4.6 mm.

The first measurements were performed using a standard method of the Pharmaceutical Biotechnology Group. Solvent A was distilled water with 0.1% (V/V) of trifluoroacetic acid (TFA) (Höninger, 2020; Boros et al., 2014), Solvent B was acetonitrile. The flow rate was set to 1 ml/min, the column oven temperature to 25 °C and the detection wavelength to 280 nm (Höninger, 2020; Boros et al., 2014).

Table 3. Gradient profile of the standard method used for HPLC.

Time (min)	Solvent A (%-V/V)	Solvent B (%-V/V)
0.0	95	5
45.0	5	95
55.0	5	95
55.1	95	5
65.0	95	5
65.1	Stop	Stop

4.10.2. Metabolite identification & quantification

The program used for the evaluation of the measured data was LabSolutions (version 5.97; Shimadzu Corp.). The peaks were compared to the external standard solution (see above) in regards to their retention time, UV-spectrum and peak profile. The integration of the peaks was performed manually, valley to valley. For the quantification of the received peak areas calibration curves of the standards had to be established in the beginning. Similar to Höninger (2020) the concentrations were set to 500, 100, 50, 10 and 5 µg/ml for arbutin and 500, 100, 50, 10, 5 and 1 µg/ml for bergenin. The preparation of the samples was alike to the procedure described above for the standard solution, however this time 1 mg/ml of each substance was dissolved separately in methanol and diluted accordingly. The injection volume was 5 µl. The measured peak areas and the corresponding concentrations were then used to create calibration curvesin Microsoft Excel 2013.

The values gained from these measurements were furthermore used to determine the limit of detection (LOD) and limit of quantification (LOQ) for the two substances – according to the ICH guideline Q2(R1) step 5 (https://www.ema.europa.eu/en/ich-q2-r1-validation-analytical-procedures-text-methodology).

For the statistical evaluation of the measured results from the elicitation experiments the software STATISTICA (version 6.1.; StatSoft Europe GmbH) was used, applying one- and two-way ANOVA followed by Duncan's Multiple Range Test ($p \leq 0.05$) for the recognition and judgement of statistically significant differences.

5. Results

5.1. Establishment of cell suspension cultures

The differences in the effects of the tested media 2 – 2d were rather slight, but noticeable (fig. 5-7). Medium 2d resulted in a fast growth but caused significant aggregate formation mostly without single cells. Media 2 and 2b were rather similar even though 2b initially seemed to produce more single cells and small cell aggregates (in the following summed up as “single cells”). Medium 2a led to a slightly slower growth with an equal amount of single cells, and medium 2c, lastly, produced (overall) the most single cells, however it also resulted in a rather slow growth.

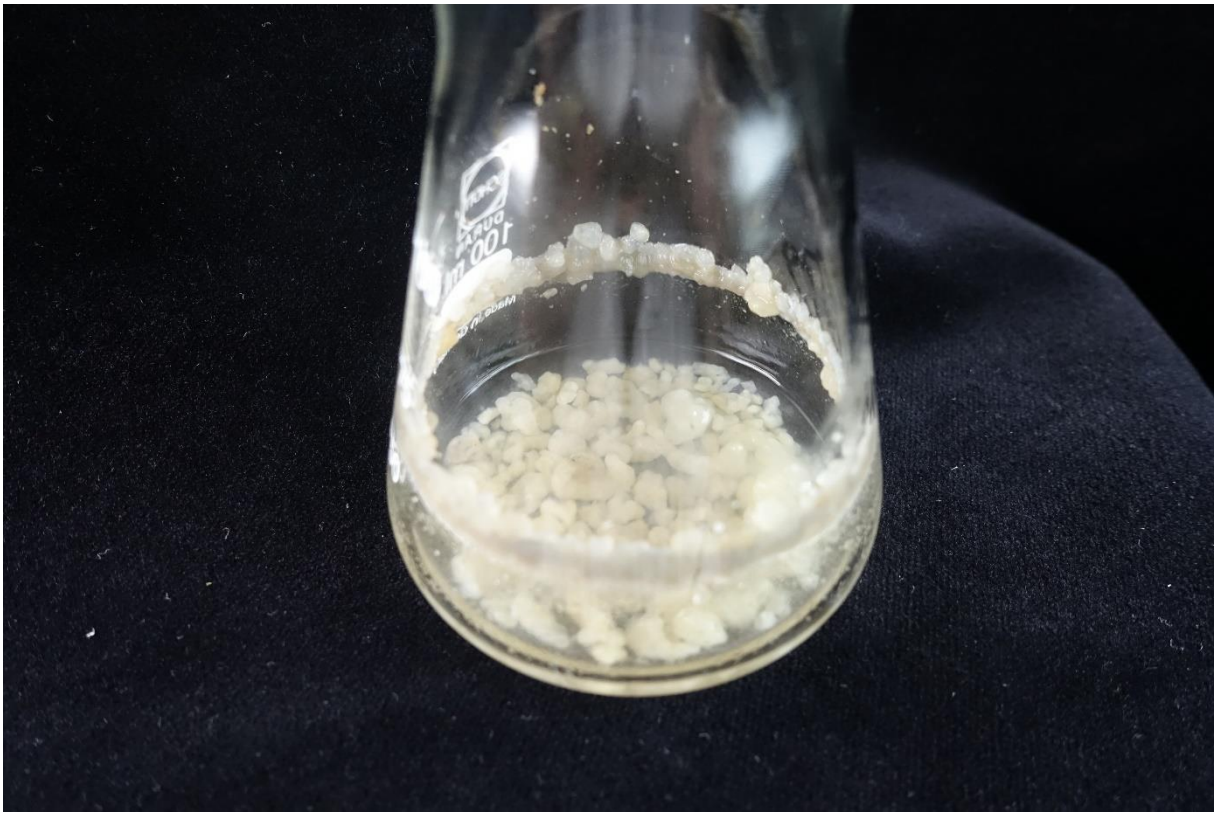

Figure 5. Suspension culture of line BP 179 in medium 2d two weeks after inoculation. The majority of callus formed big aggregates.

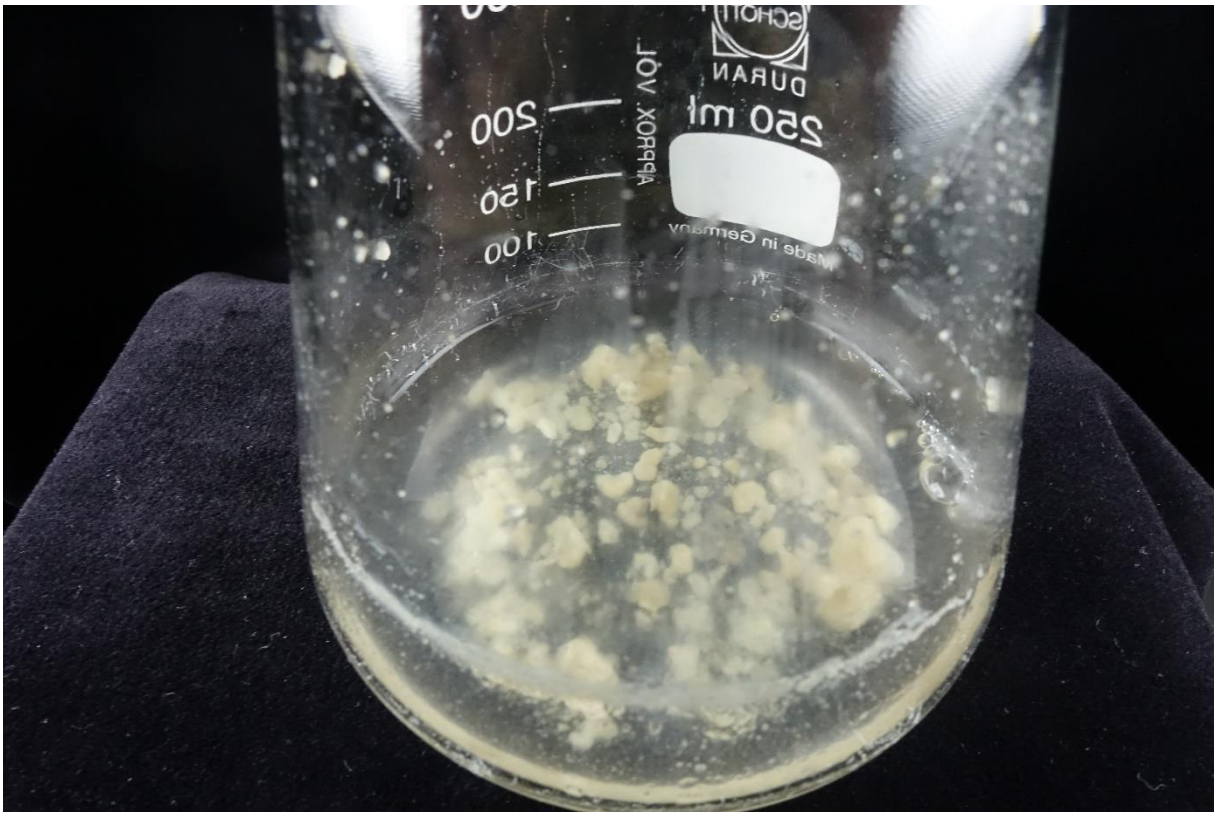

Figure 6. Suspension culture of line BP 179 in medium 2c two weeks after inoculation. Although single cells and small aggregates were formed, growth was comparably slow.

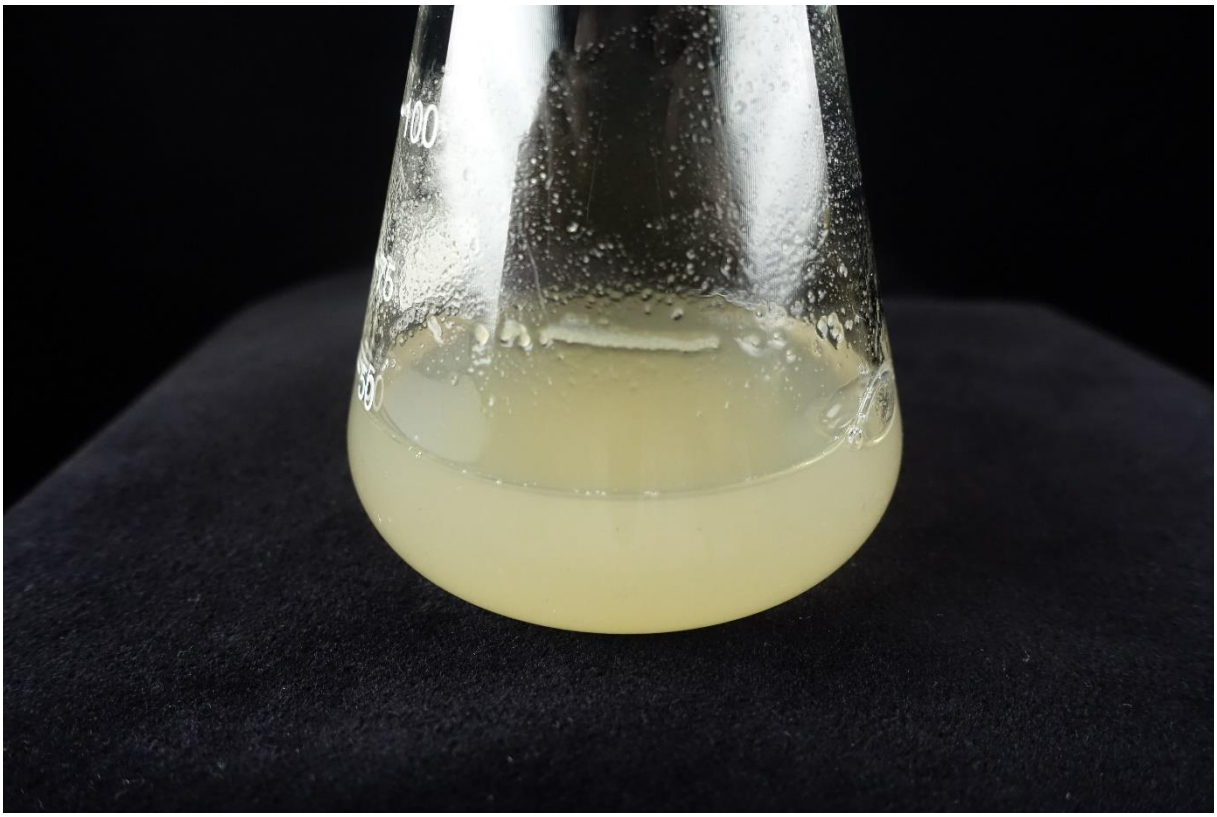

Figure 7. Suspension culture of an aliquot of sieved single cells of line BP 179 in medium 2b one week after inoculation.

Due to the obvious negative impact of media 2e – 2h (the cells turned almost completely brown or black within a few days), they have only been tried once for the cell line BP 57.

Sieving of the cultures in the different media and pipetting an aliquot of sedimented cells, as described above, generally resulted in one new inoculated flask per 5 sieved flasks but the sieved cultures didn't show any growth, most likely because of low cell density (see below).

Further attempts using pectinase led to the desired result (i.e. a decrease of big aggregates and formation of small aggregates and single cells); however, the treatment caused a large amount of cells (both in aggregates and single cells) to die as well.

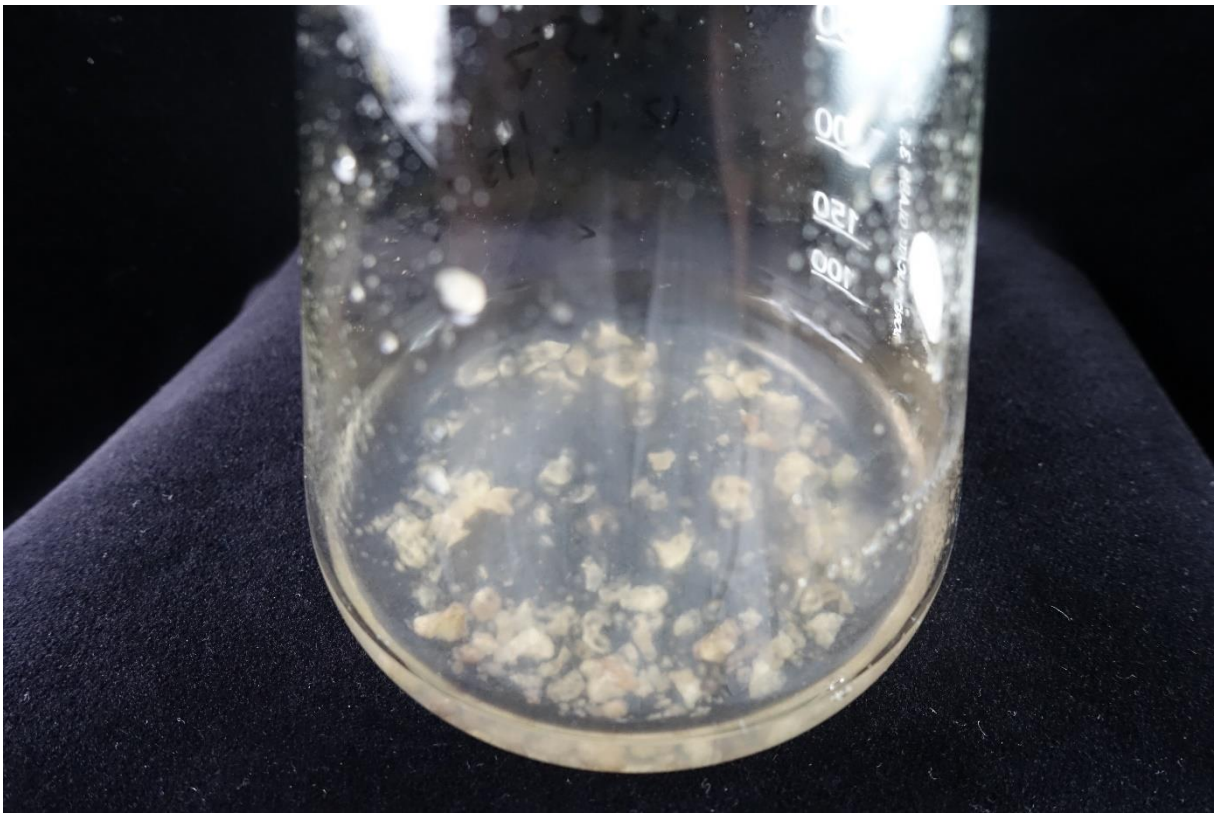

Figure 8. Suspension culture of line BP 57 in medium 2a one week after addition of pectinase – noteworthy is the cloudy character of the medium due to the amount of single cells; the remaining aggregates are small of rather brown color.

Further attempts with lower concentrations of pectinase (namely 200 µl of the stock solution per 50 ml medium, as well as 100 µl in 50 ml) only showed mediocre or almost no effects – in that the amount of (living) single cells produced was higher, compared to medium-only cultures, however the remaining aggregates were almost unusable and the amount of available cells for future subculturing therefore in total lower.

These experiments showed, that in most cases there was only a small portion (varying from 20 to 50%) of living single cells. Attempts to gently centrifuge the sieved single

cells at 400 rpm for 10 minutes and resuspend the gained pellet in only 10 ml fresh medium to obtain a higher cell density didn't lead to better results.

The next experiment – blending of callus aggregates from semi-solid medium with liquid medium 2, as described above – led to very small aggregates as well as a reasonable amount of single cells. The major disadvantage of this method was the occurrence of microbial contaminations in the resulting cultures: the blender couldn't be autoclaved due to its plastic seal, and the disinfection with 70 Vol.-% ethanol was apparently not effective enough.

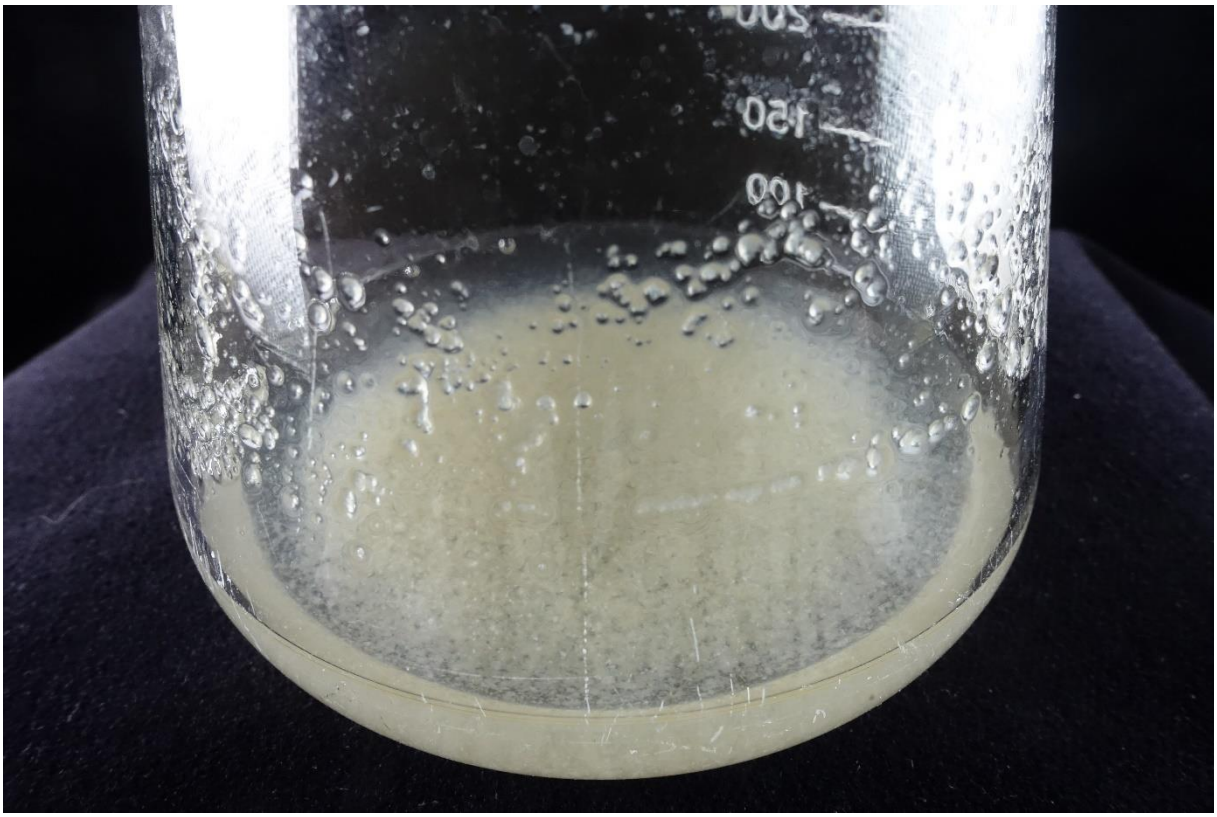

Figure 9. BP 179 in medium 2 one day after blending.

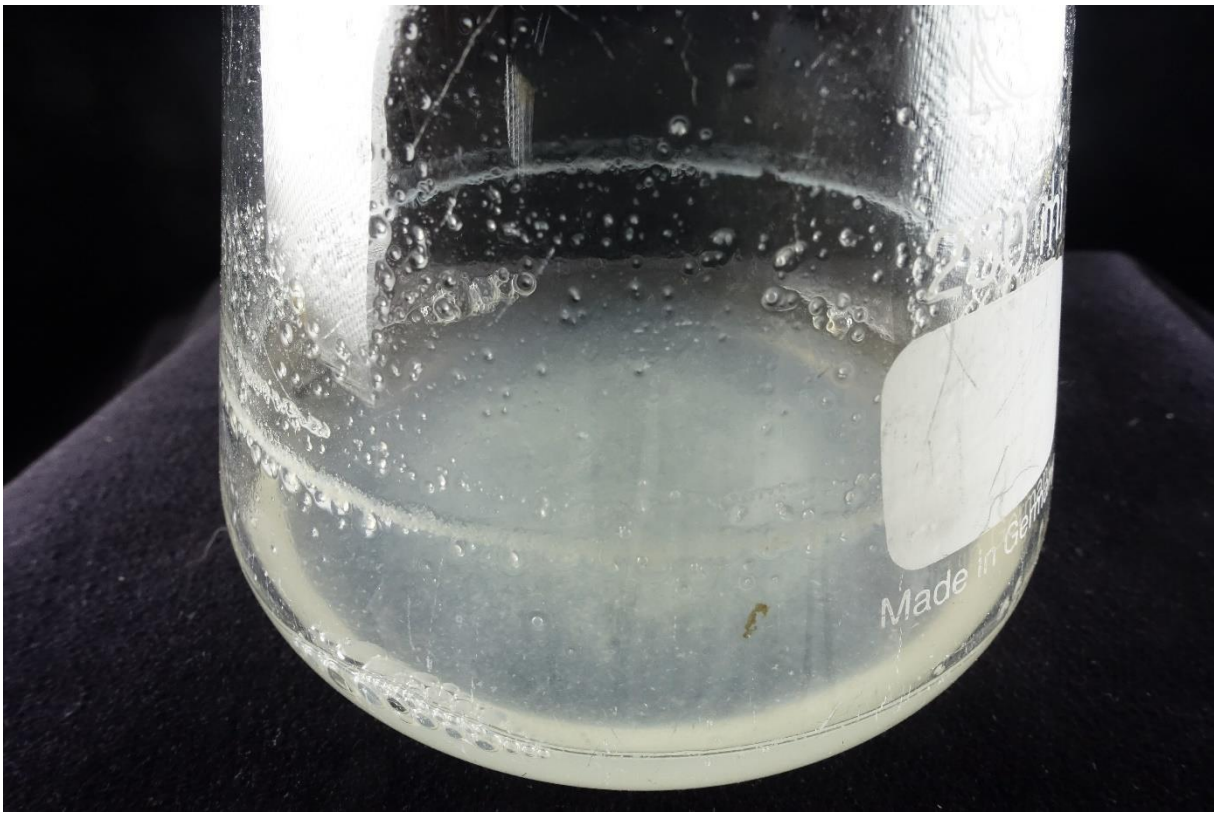

Figure 10. Sieved single cells of the same culture and medium, one day after blending as well.

Finally, the mechanical method using glass beads to disrupt cell aggregates has been tried. Here both cultures with and without glass beads have been compared in regards to the effectivity, size of the glass beads and the amount added, as well as the rotary shaker speed (150 rpm and 200 rpm). The first experiments (3 g or 4 g of 2 or 3 mm glass beads at 150 rpm) were conducted using 5 flasks per callus line, the later ones with only 3 flasks each. Since only of lines BP 57 and BP 179 a sufficient amount of callus was available at this time, only these two were tested.

Since the first attempts showed a faster darkening of the cultures, 1 ml of a 50 mg/ml PVP-10 (Sigma-Aldrich, PVP10) stock solution (in order to create a 0.1% solution within the media; cf. Malla, 1999) was added, to absorb possible phenolic compounds.

The 2 mm glass beads showed almost no effect (neither 3 nor 4 g). The increased speed of the rotary shaker produced more single cells in all cultures (compared to the standard speed of 120 rpm). Using 3 mm glass beads resulted in an increased amount of single cells, there was however no visible difference between 3 or 4 g, and the increase of the shaking speed from 150 to 200 rpm didn't show a great effect. The addition of the PVP-10 solution showed the greatest effect on the cultures without glass beads at high speed; the effects on the cultures with glass beads were only slight. Ultimately this treatment

wasn't very satisfying as the amount of living single cells was rather low (about 50%) and the effect on the callus aggregates due to the mechanic impact led to a smaller amount of useable material for subculturing (compared to untreated cultures).

Since the aforementioned methods were at last not useable, it was decided to finely chop the callus aggregates selected for the elicitation experiments in order to obtain small aggregates with 2-3 mm diameter on average.

5.2. Growth curves of cell suspension cultures

Two of the five available culture lines, namely BP 57 and BP 179, were chosen for the establishment of growth curves. The main criterium for this selection was the general availability of enough callus material. Additionally, aggregate formation in line BP 179 was comparatively low.

The growth was estimated on the one hand by calculating the volume derived from the taken height measurements of the sedimented callus cells – for which a straight calibration line, using defined volumes and the corresponding scale values, was established (fig. 11) – on the other hand the inoculation weight was compared to the fresh weight (measured after filtration through a Büchner funnel for 5 minutes) at the end of the growth curve and compared to the results of the volumetric values (and scale measurings alike).

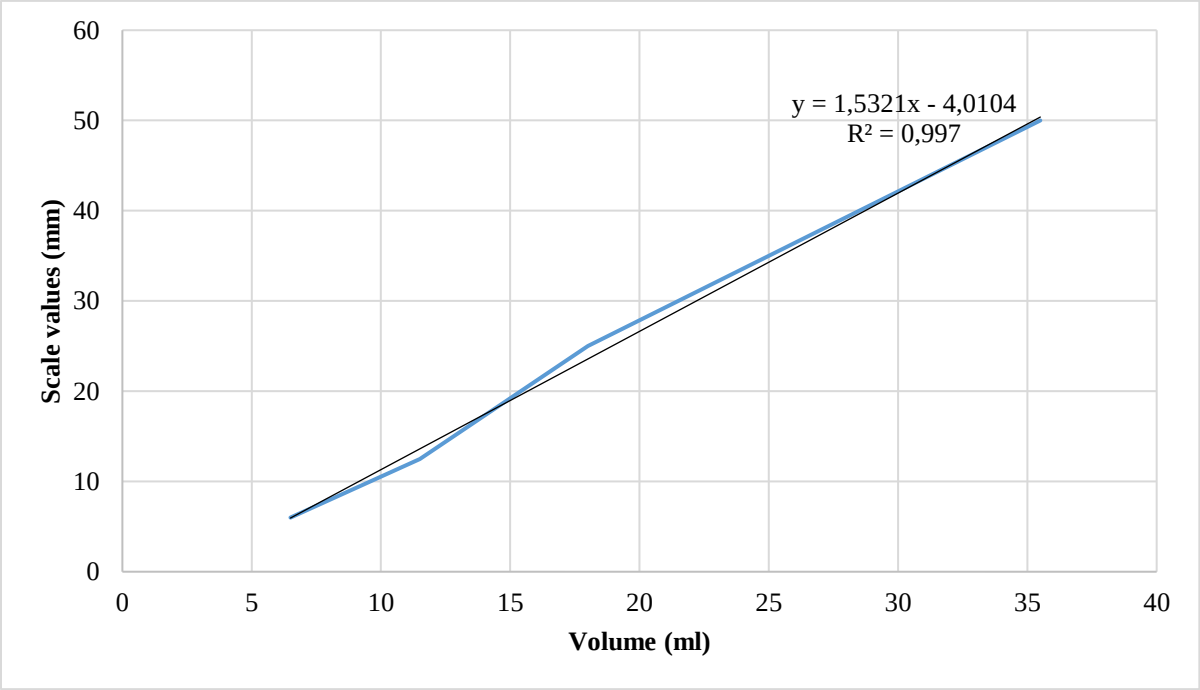

Figure 11. Straight calibration line for volume measurements (blue; regression line in black); linear equation and correlation coefficient are given as well.

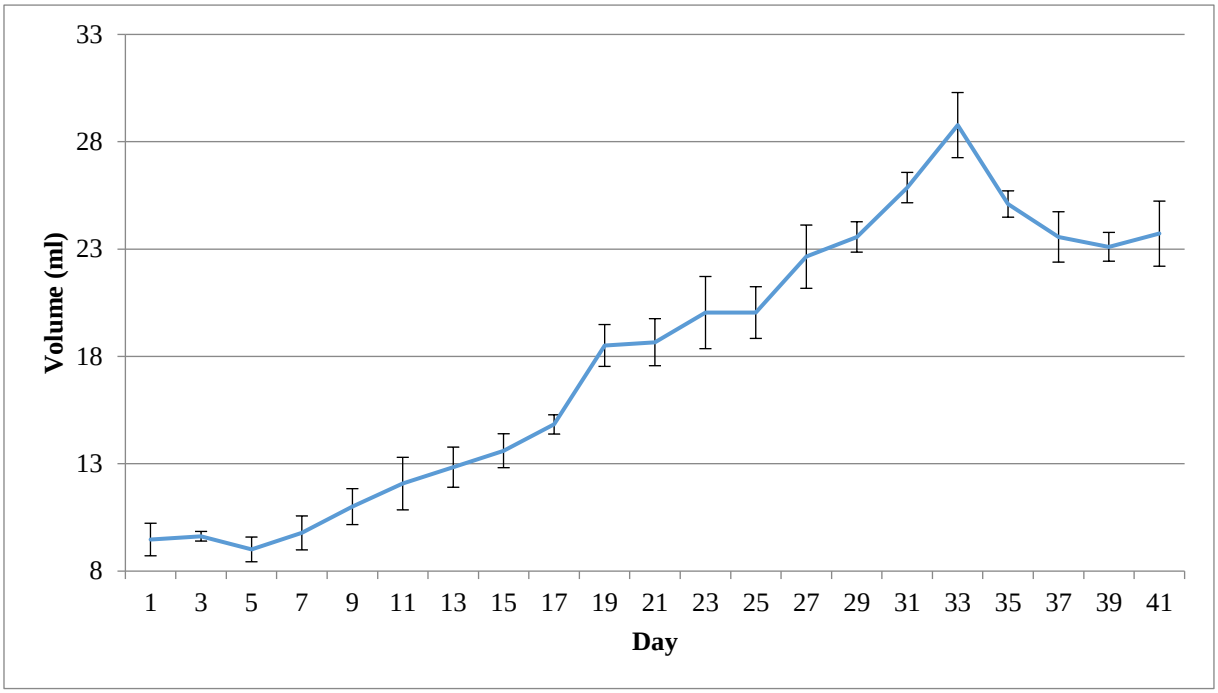

Figure 12. Growth curve of line BP 57. Means and standard deviation for each measuring point (n = 5) are shown on the graph.

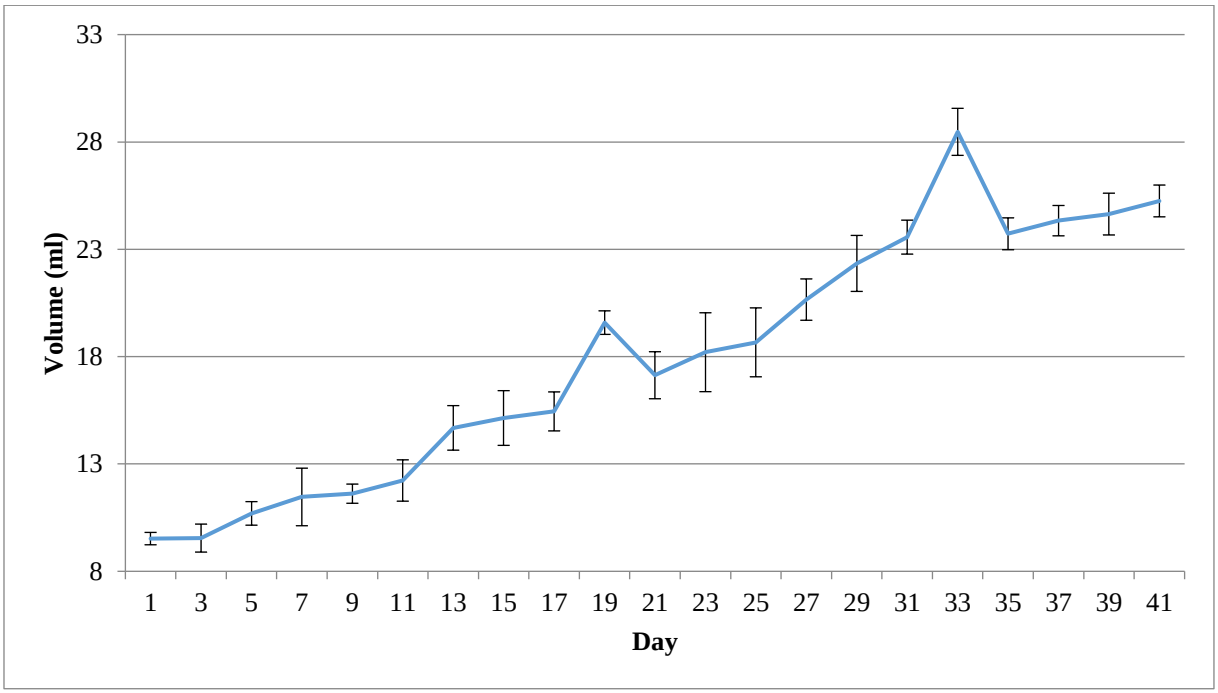

Figure 13. Growth curve of line BP 179. Means and standard deviation for each measuring point (n = 5) are shown on the graph.

The average inoculation weight of BP 57 was 4.0612 ± 0.0680 g and the average fresh weight on day 41 was 13.4764 ± 1.0663 g, and there was a clear correlation between these weights and the scale values and estimated volume. The average inoculation weight of BP

179 was 4.0274 ± 0.0286 g, the average fresh weight at day 41 was only 4.8942 ± 0.6906 g, which didn't correlate with the volume at day 41 (which was approx. 2.5-fold of the initial volume). The reason for this phenomenon is not known.

Based on the growth curves the timepoint for elicitation was set at day 17.

5.3. Identification, classification and selection of endophytes

The results of the identification process, which was conducted on the one hand to gain information about the cultivable endophytes colonizing *B. pacumbis*, on the other hand to find suitable individuals for the subsequent elicitation, can be seen in Table 4, which shows the most similar sequence matches of the consensus sequences resulting from the assembled forward and reverse sequences of the 16S rDNA based on the database of EzBioCloud (Yoon et al., 2017).

Table 4. Most similar taxa to experimental endophyte sequences referenced with curated EzBioCloud 16S database (Yoon et al., 2017). PS – pooled sample; R – root; L – leaf; P – petiole. "Completeness" refers to the sequence between 27F and 1492R primers (Kim et al., 2012; Lane, 1991). Informations regarding the plant- and tissue-origin of isolates were provided by Dr. Oberhofer.

Isolate ID	Most similar taxon	Plant	Replicate	Tissue	Similarity (%)	Completeness (%)
BL 21	*Pseudomonas hunanensis*	2	1	R	99.50	97.9
BL 47	*Xanthomonas melonis*	1	3	L	99.51	97.5
BL 65	*Micrococcus luteus*	PS	PS	P	99.36	97.9
BL 67	*Micrococcus luteus*	PS	PS	P	99.50	98.1
BL 79	*Prolinoborus fasciculus*	-	-	P	99.64	98.2
BL 80	*Lelliottia jeotgali*	2	2	R	99.40	98.0
BL 82	*Prolinoborus fasciculus*	PS	PS	P	99.57	98.3
BL 91	*Micrococcus luteus*	PS	PS	P	99.15	98.1
BL 92	*Prolinoborus fasciculus*	PS	PS	P	99.71	97.8
BL 94	*Bacillus cereus*	PS	PS	P	99.51	97.6
BL 96	*Moraxella osloensis*	PS	PS	P	98.60	98.0
BL 99	*Janibacter indicus*	PS	PS	P	99.22	97.8
BL 100	*Micrococcus luteus*	PS	PS	P	99.65	97.9
BL 103	*Prolinoborus fasciculus*	PS	PS	P	99.42	98.2
BL 106	*Micrococcus luteus*	PS	PS	P	99.08	98.4

BL 107	*Micrococcus luteus*	PS	PS	P	99.58	98.5
BL 109	*Janibacter indicus*	PS	PS	P	99.43	97.9
BL 110	*Staphylococcus capitis subsp. urealyticus*	PS	PS	P	99.51	97.9
BL 116	*Janibacter indicus*	PS	PS	P	99.65	98.1
BL 117	*Paracoccus yeei*	PS	PS	P	99.26	98.1
BL 121	*Prolinoborus fasciculus*	PS	PS	P	99.64	98.2
BL 125	*Paracoccus aerius*	PS	PS	P	99.63	97.9
BL 137a	*Pseudomonas alloputida*	2	1	R	99.79	97.7
BL 138	*Micrococcus luteus*	PS	PS	P	99.43	97.7
BL 141	*Micrococcus luteus*	PS	PS	P	99.50	98.5
BL 142	*Paracoccus aerius*	PS	PS	P	99.41	97.9
BL 144	*Microbacterium foliorum*	3	2	P	99.16	98.3
BL 147	*Pseudomonas sp.*	2	3	P	98.74	98.0

For the following three species (table 5) only the reverse sequence has been used for database screening.

Table 5. Most similar taxa to experimental endophyte sequences referenced with curated EzBioCloud 16S database (Yoon et al., 2017). PS – pooled sample; R – root; L – leaf; P – petiole. "Completeness" refers to the sequence between 27F and 1492R primers (Kim et al., 2012; Lane, 1991). Informations regarding the plant- and tissue-origin of isolates were provided by Dr. Oberhofer.

Isolate ID	**Most similar taxon**	**Plant**	**Replicate**	**Tissue**	**Similarity (%)**	**Completeness (%)**
BL 76	*Pseudomonas alcaligenes*	2	3	P	98.78	56.1
BL 86b	*Acinetobacter lwoffii*	PS	PS	P	99.52	85.4
BL 113	*Paracoccus panacisoli*	PS	PS	P	99.58	51.4

The resulting unrooted phylogenetic tree using the MEGA X software (as described above) can be seen in figure 14.

The subsequent statistical and work-process details were automatically generated by the software MEGA X as output together with the phylogenetic tree; they were adopted in this form with slight modifications: The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-7282.53) is shown. The percentage of trees in which the

associated taxa clustered together is shown next to the branches (bootstrap support estimated on 500 replicates). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3468)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 47 nucleotide sequences. There were a total of 1314 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

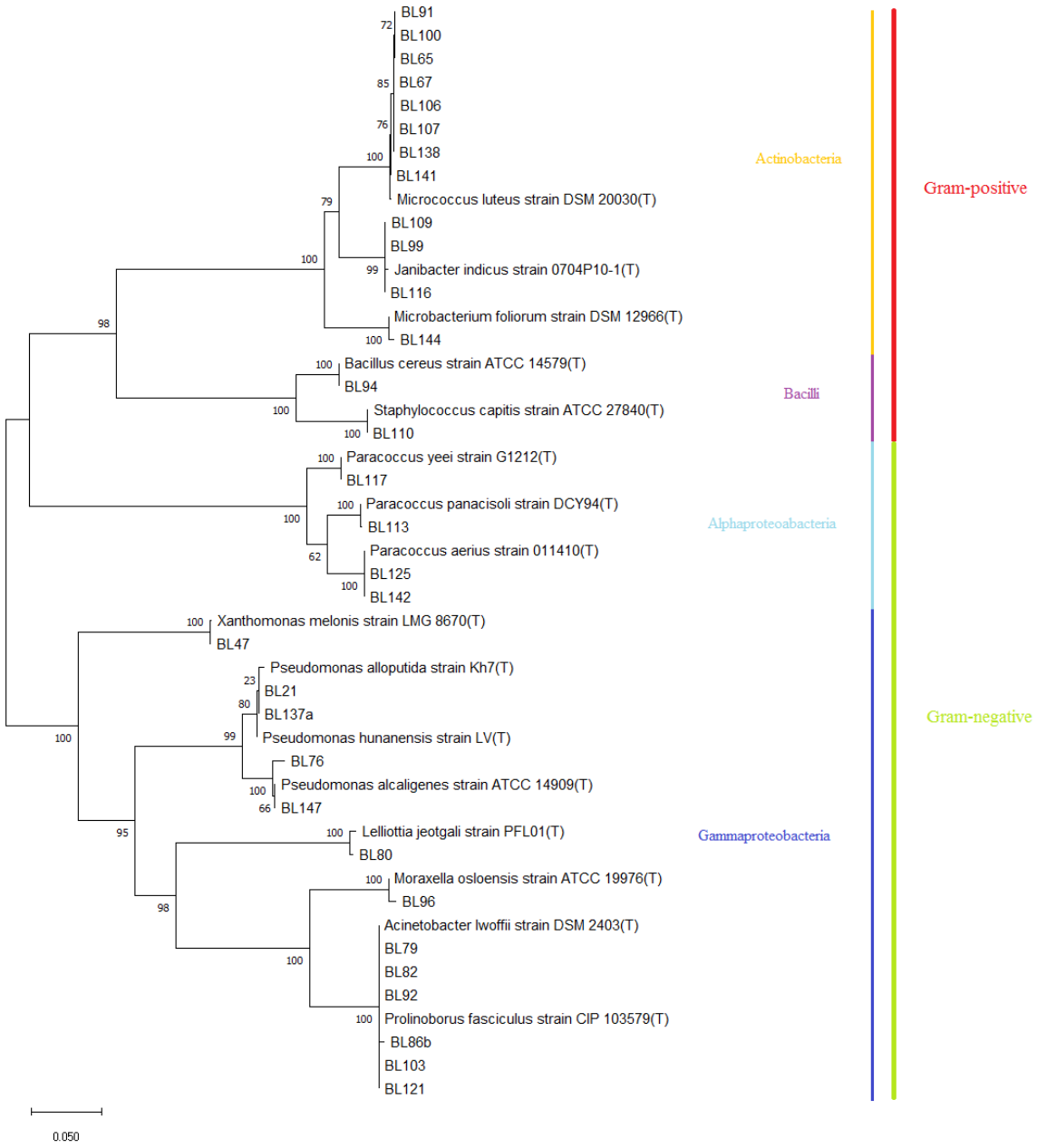

Figure 14. Maximum likelihood analysis of 16S rDNA sequences obtained from endophytes of *Bergenia pacumbis*. Trees were calculated by MEGA X using an alignment with 1314 informative sites and 500 replications for bootstrap support. Resulting clades were grouped in Gram-positive (red) and Gram-negative bacteria (green), which were subdivided into their respective classes – Actinobacteria (orange) and Bacilli (purple) on the one hand, and Alpha- (light-blue) and Gammaproteobacteria (dark-blue) on the other hand.

The selection of the endophytes for elicitation was carried out in regards to their phylogenetic diversity and/or the known ability to produce interesting metabolites, as mentioned above; the five species used are shown in Table 6.

Table 6. Endophyte species used for elicitation (sequencing results according to EzBioCloud).

Isolate ID	Sequencing result	Phylogenetic characteristics
BL 96	*Moraxella osloensis*	G-; Gammaproteobacterium
BL 117	*Paracoccus yeei*	G-; Alphaproteobacterium
BL 137a	*Pseudomonas alloputida*	G-; Gammaproteobacterium
BL 141	*Micrococcus luteus*	G+; Actinobacterium
BL 144	*Microbacterium foliorum*	G+; Actinobacterium

5.4. Bacterial growth curves

The growth measurements were conducted as described above (see chapter 4.8.2.). The rather fast growth of certain endophytes caused some growth curves to result in a 'fragmentary' character (i.e. only the last part of the actual growth phase as well as stationary and death phase were measured). The results of the measurments (described in hours post inoculation for the two respective media) can be found in table 7. Due to a technical error while converting the gained results into an Excel-sheet, the OD values for BL 137a, 141 and 144 24 h post inoculation are not available, instead a fourth measurement (sc. 30 hours post inoculation) was conducted for these three strains; measurings from the following time points are the same for all five strains. Examples for established growth curves (using Microsoft Excel 2013) are shown in figures 15 and 16.

Table 7. Endophyte species used for elicitation (taxonomic classification according to EzBioCloud).

Isolate ID	Sequencing result	Estimated time of stationary phase (hours post inoculation) in respective medium
BL 96	*Moraxella osloensis*	48 (LB) / 50 (TSB)
BL 117	*Paracoccus yeei*	72 (LB) / 26 (TSB)
BL 137a	*Pseudomonas alloputida*	30 (LB) / 48 (TSB)
BL 141	*Micrococcus luteus*	48 (LB) / 50 (TSB)
BL 144	*Microbacterium foliorum*	30 (both)

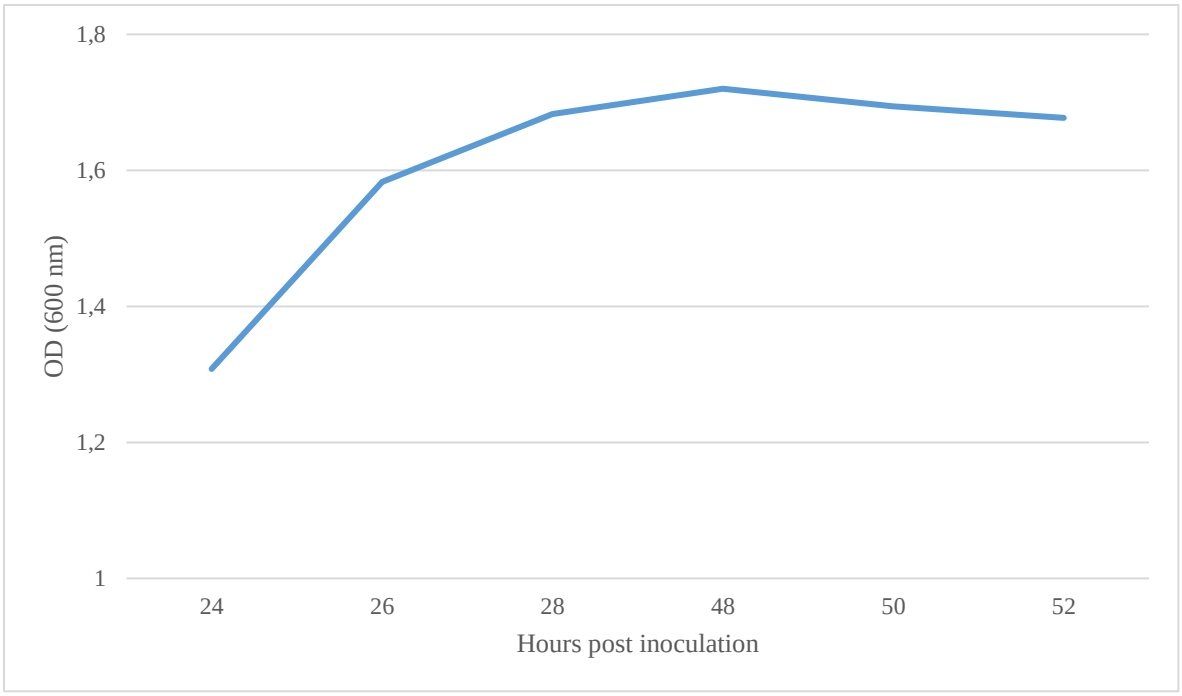

Figure 15. Growth curve of endophyte BL 96 in LB medium.

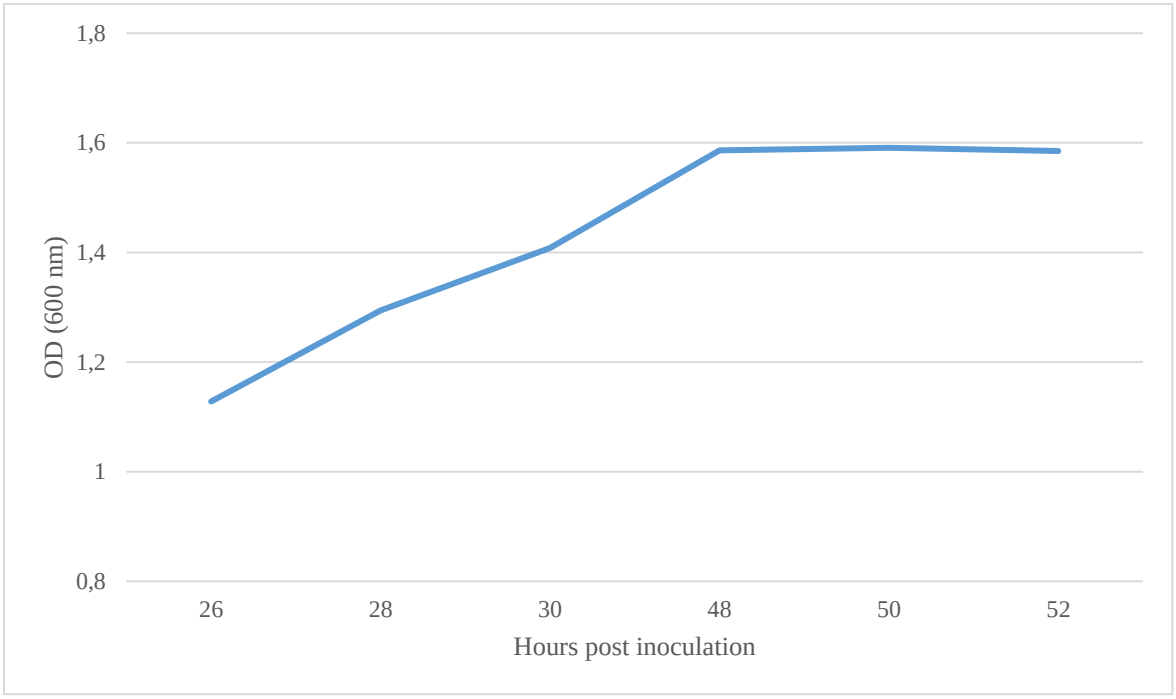

Figure 16. Growth curve of endophyte BL 141 in TSB medium.

5.5. HPLC method optimization

The standard HPLC method which was used initially showed good results, however it had to be modified mainly in regards to the duration (no substances were detected after minute 25). Additionally, two minutes of flushing with water/TFA were added at the beginning of the elution to prevent highly hydrophilic substances (especially sugars) from overlapping with the subsequent peaks (Tahir, personal communication).

Table 8. Gradient profile of the optimized HPLC method.

Time (min)	Solvent A (%-V/V)	Solvent B (%-V/V)
0.0	100	0
2.0	100	0
15.0	5	95
25.0	5	95
25.1	100	0
35.0	100	0
35.1	Stop	Stop

5.6. HPLC calibration curves

The measurements for the calibration curves were carried out as described (chapter 4.10.2.). The exact initial weight for the standards were 0.98 mg for arbutin and 1.07 mg for bergenin. The resulting exact concentrations (in the given order) were therefore: 490, 98, 49, 9.8 and 4.9 µg/ml for arbutin and 535, 107, 53.5, 10.7, 5.35 and 1.07 µg/ml for bergenin.

The measured peak areas and the corresponding concentrations were used to create straight calibration lines in Microsoft Excel 2013. The results, including the linear equation, correlation coefficient and a regression line can be seen in figures 17 and 18.

The resulting LOD values are 5.40 µg/ml for arbutin and 1.96 µg/ml for bergenin, the results for LOQ 16.36 µg/ml (arbutin) and 5.95 µg/ml (bergenin).

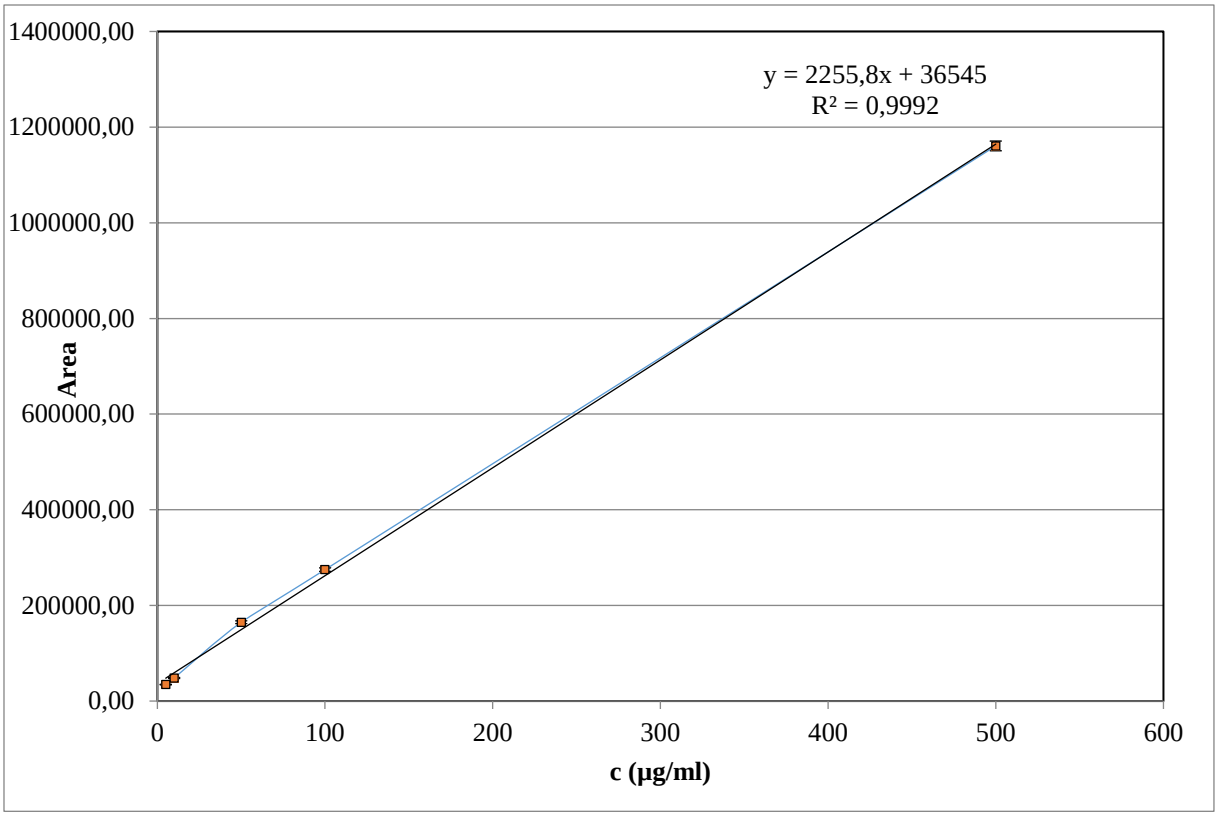

Figure 17. Straight calibration line (blue) for arbutin. Means with standard deviations (n = 3), linear equation and correlation coefficient as well as regression line (black) are depicted.

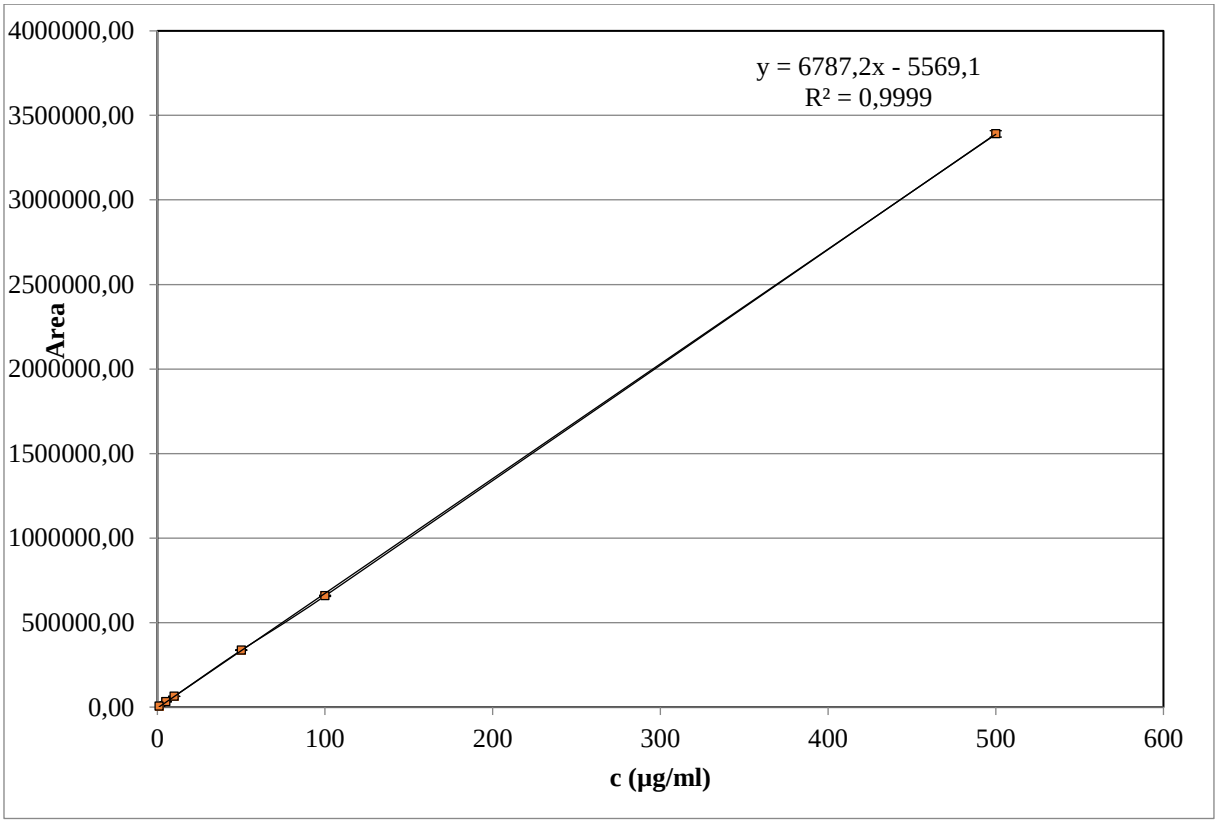

Figure 18. Straight calibration line for bergenin. Means with standard deviations (n = 3) as well as linear equation and correlation coefficient are depicted. Due to high homology of the straight calibration line and the regression line, the optical difference is not visible here.

5.7. Metabolite identification & quantification

For a better comparison of the peak areas measured in the treated cultures, the initial standard concentration of 500 µg/ml each was decreased to 100 µg/ml (fig. 19), and the identification was done as described above. The extracts of the lyophilized suspension culture media showed an absence of both arbutin and bergenin, while in the extracts of lyophilized callus cells only bergenin could be detected.

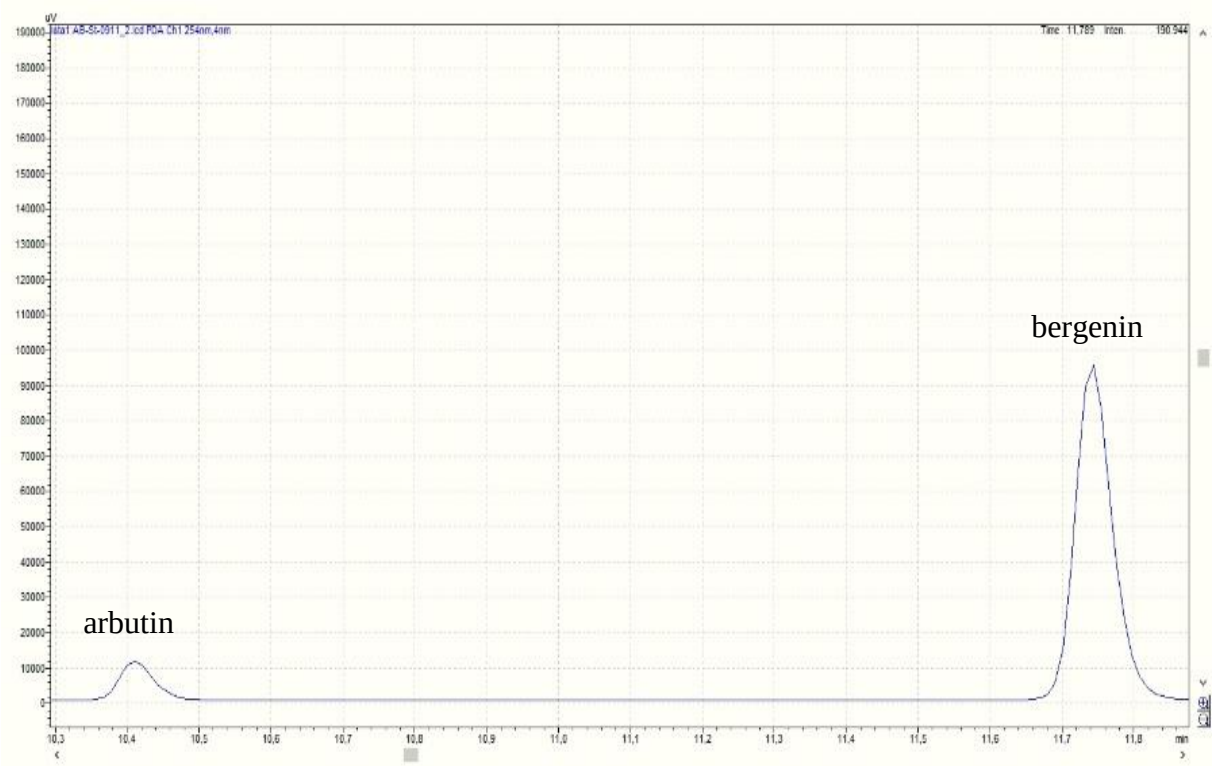

Figure 19. Chromatogram of the combined standards arbutin and bergenin at a concentration of 100 µg/ml each.

The measured peak areas were the basis for the calculation of the bergenin concentrations (both in mg/g and %-m).

The endophytes utilized for the elicitation and depicted in the diagrams are, like mentioned before (chapter 5.4.), *Moraxella osloensis* (BL 96), *Paracoccus yeei* (BL 117), *Pseudomonas alloputida* (BL 137a), *Micrococcus luteus* (BL 141) and *Microbacterium foliorum* (BL 144).

The first set of diagrams shows the amounts of bergenin produced in cell line BP 57 when treated with resuspended autoclaved bacterial cells, on the one hand, and sterile filtrated LB or TSB medium of the respective bacteria lines, on the other hand (fig. 20 – 23).

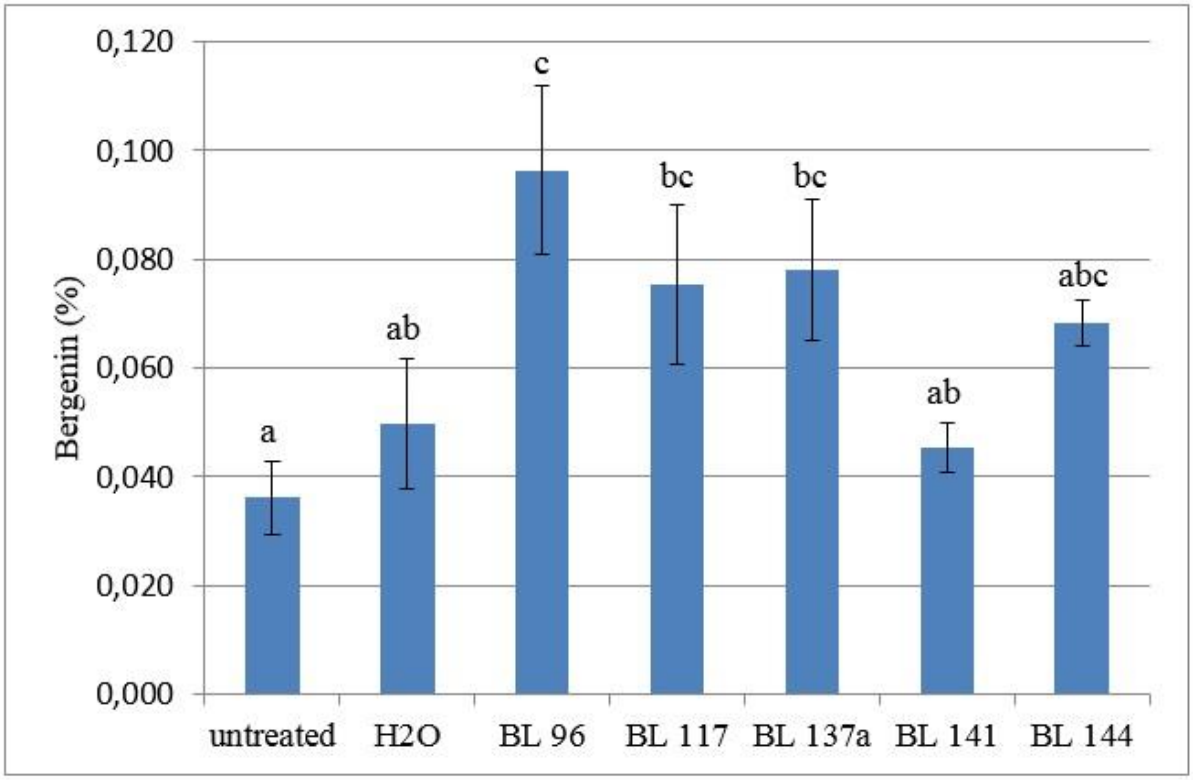

Figure 20. Amount of bergenin in cell line BP 57 elicited with bacterial cells cultivated in LB medium, compared to untreated cultures and water-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

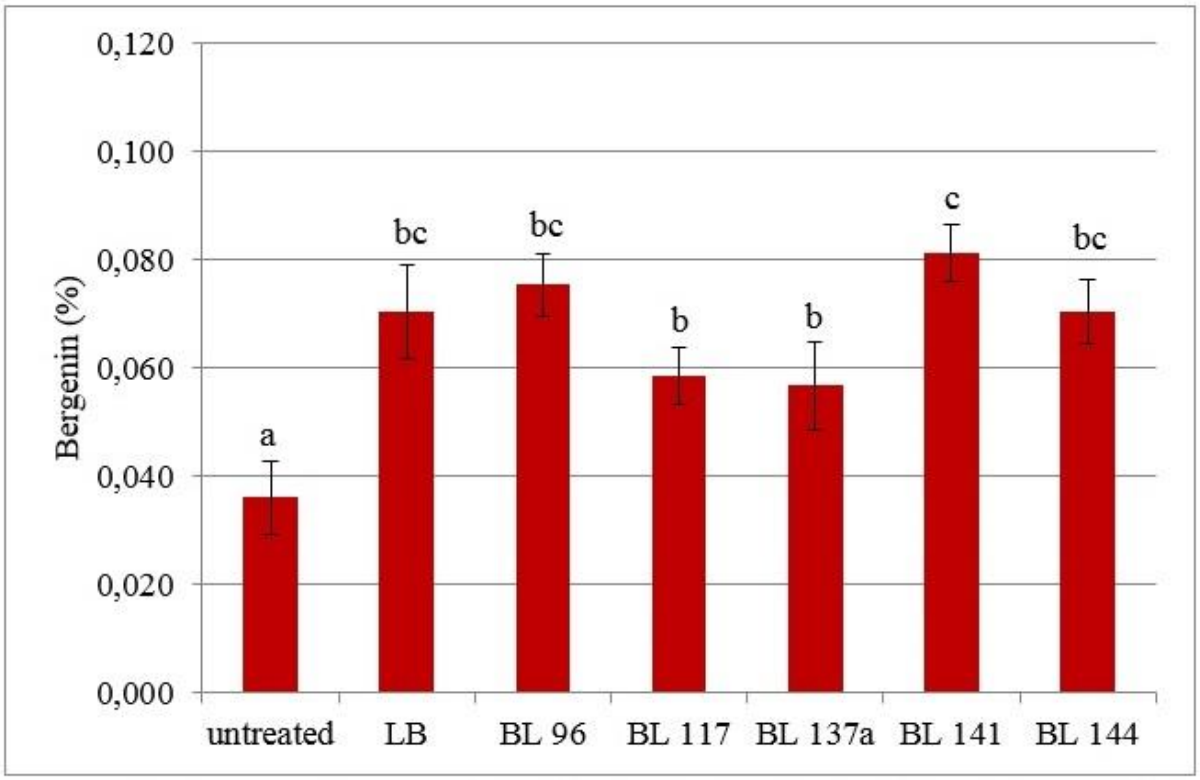

Figure 21. Amount of bergenin in cell line BP 57 elicited with sterile-filtrated LB medium of the respective bacteria, compared to untreated cultures and LB-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

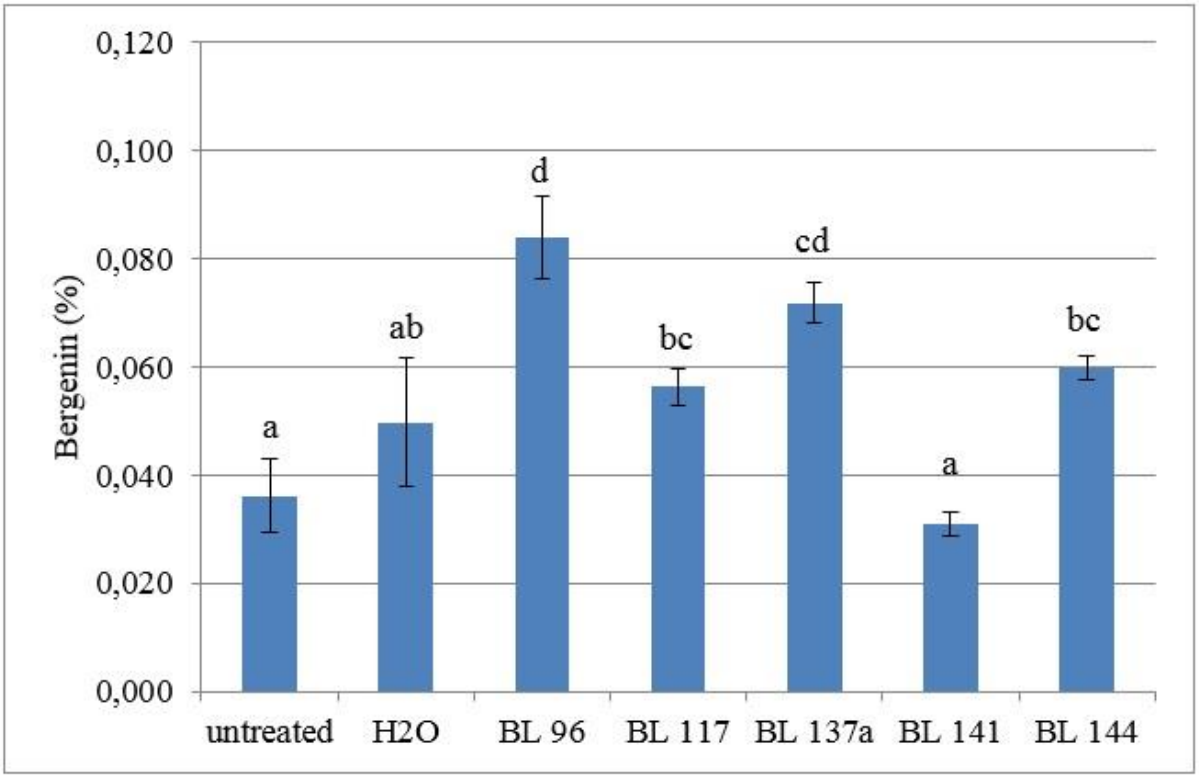

Figure 22. Amount of bergenin in cell line BP 57 elicited with bacterial cells cultivated in TSB medium, compared to untreated cultures and water-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

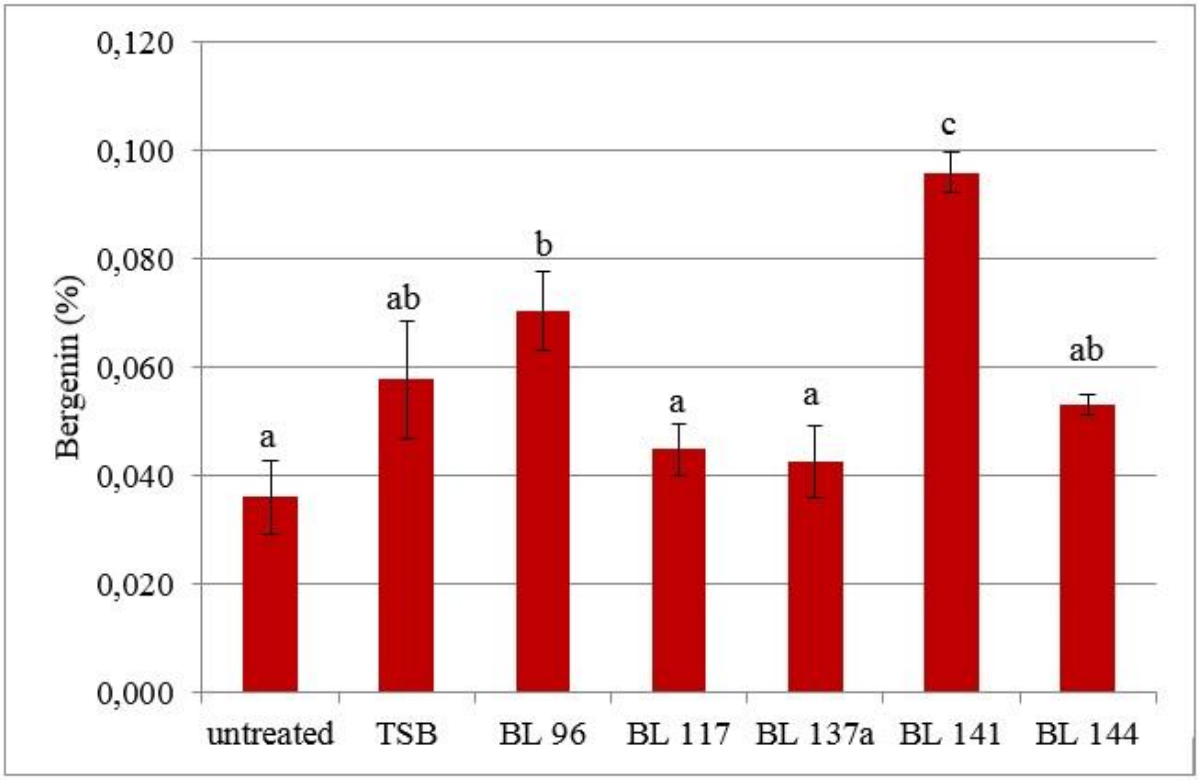

Figure 23. Amount of bergenin in cell line BP 57 elicited with sterile-filtrated TSB medium of the respective bacteria, compared to untreated cultures and TSB-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

The next set of data (fig. 24 – 27) shows, on the one hand, the different influence of treatment with bacterial cells compared to sterile-filtrated medium (for both LB and TSB medium) and, on the other hand, the influence of the two media (shown for both cell- and medium-treatment).

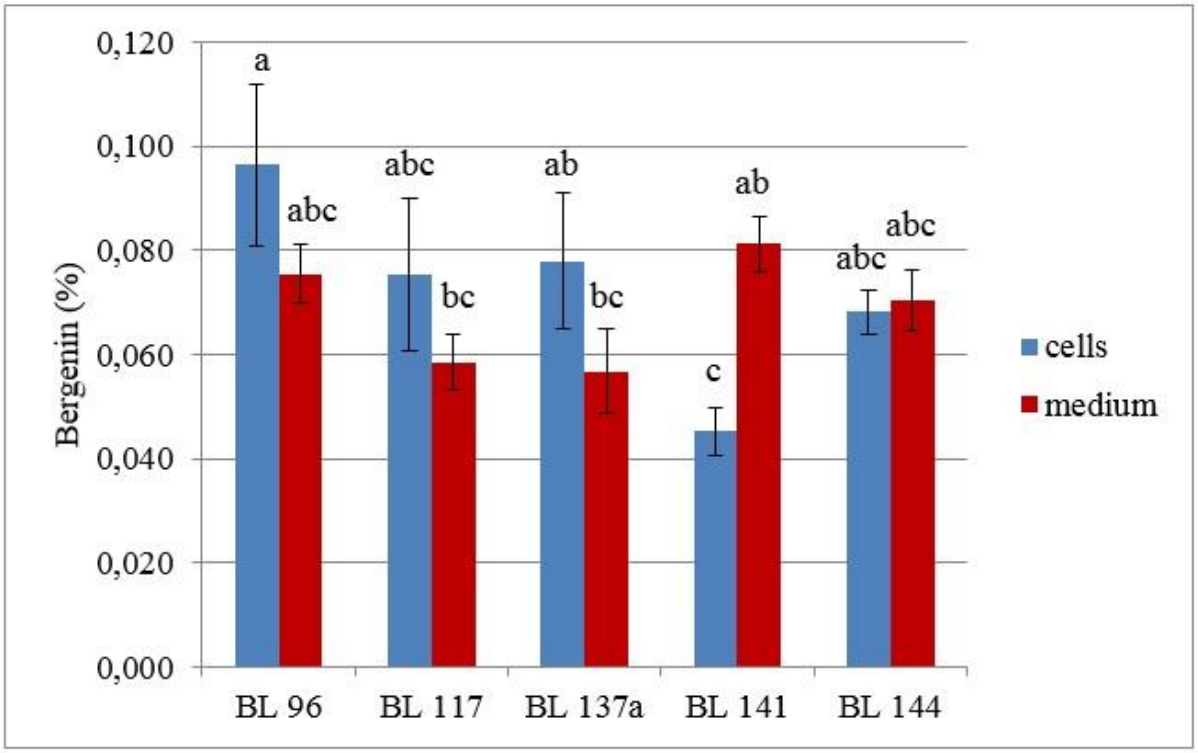

Figure 24. Amount of bergenin in cell line BP 57 elicited with either autoclaved bacterial cells grown in LB medium, or with sterile-filtrated LB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

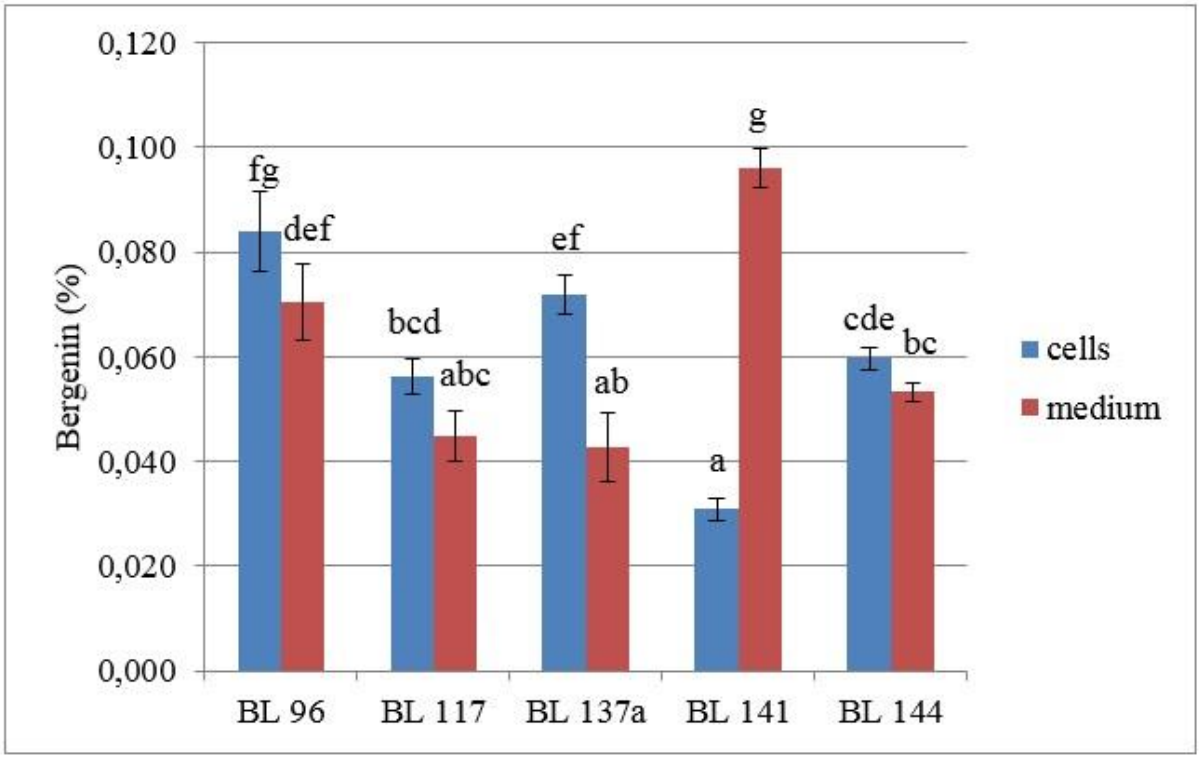

Figure 25. Amount of bergenin in cell line BP 57 elicited with either autoclaved bacterial cells grown in TSB medium, or sterile-filtrated TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

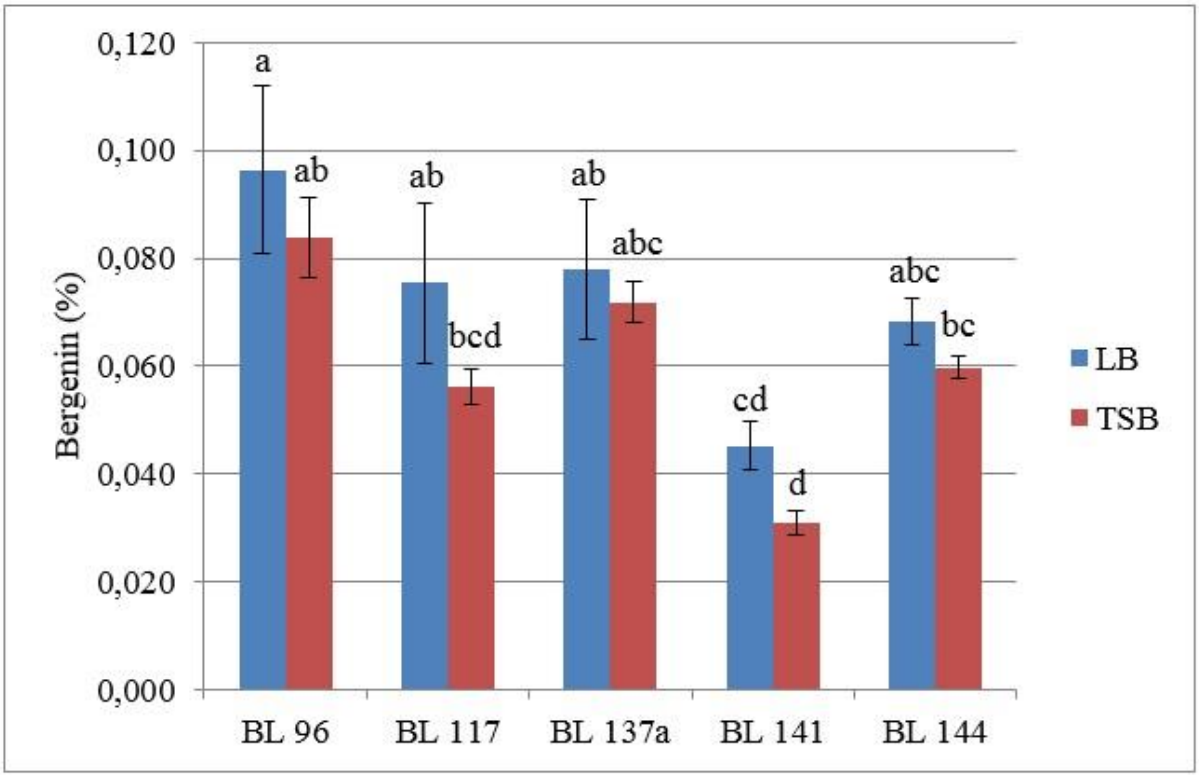

Figure 26. Amount of bergenin in cell line BP 57 elicited with autoclaved bacterial cells grown in either LB or TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

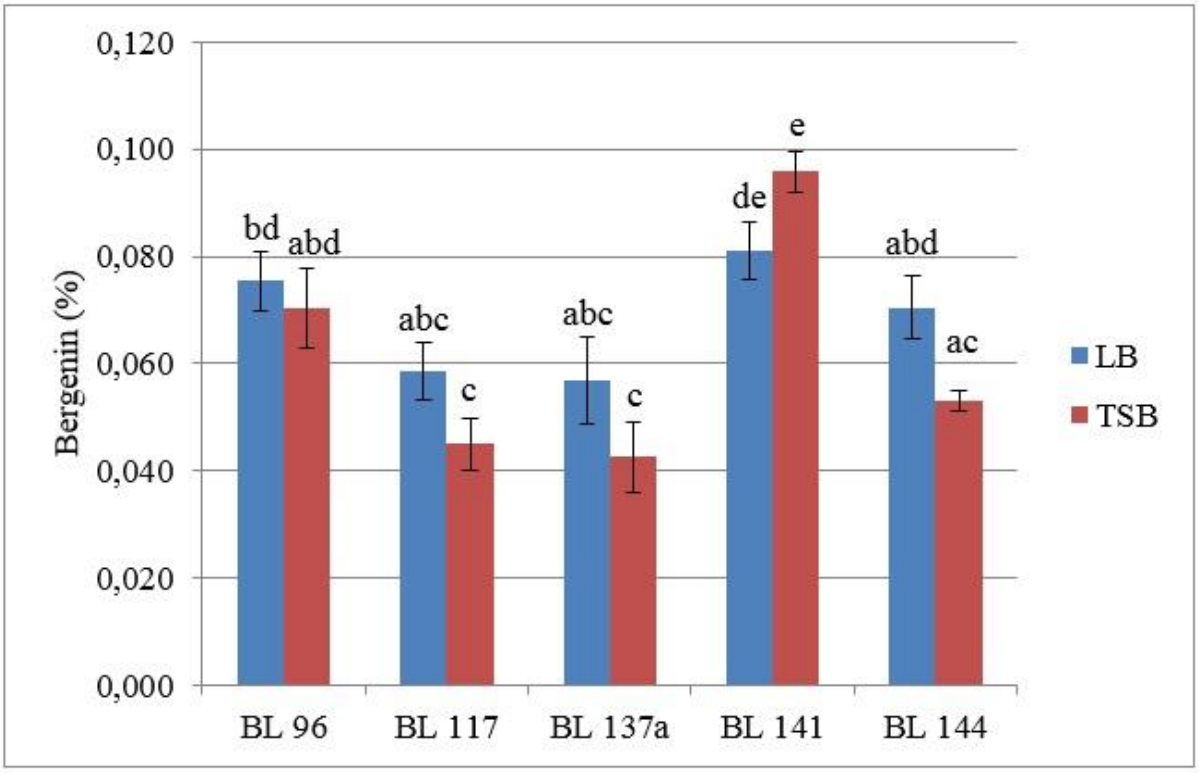

Figure 27. Amount of bergenin in cell line BP 57 elicited with sterile-filtrated bacterial LB or TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan‘s Multiple Range Test).

To summarize, there was a statistically significant increase in the produced amount of bergenin for cultures elicited with BL 96 cells (grown in LB or TSB medium) and BL 137a cells (grown in TSB medium), compared to unelicited cultures and water-control. There was furthermore a statistically significant increase in bergenin, compared to unelicited cultures, after elicitation with BL 117 cells (grown in LB or TSB medium) as well as with BL 137a cells (grown in LB medium) and BL 144 cells (grown in TSB medium). The treatment with sterile filtrated LB medium showed a significant increase for all treatments (compared to untreated), however, no difference between medium-only and bacterial media; the highest bergenin amounts were obtained upon treatment with BL 141 medium. The elicitation with BL 141 TSB medium showed a significant increase in bergenin, compared to untreated cultures and medium-only treatment. Elicitation with BL 96 TSB medium resulted in a significant increase in bergenin, compared to untreated cultures. The comparison of cell- and medium-treatment showed a significant difference for BL 141 LB and TSB elicitors, where the amount of bergenin produced after elicitation with TSB medium, compared to cells, was approximately 3 times as high. Furthermore, there was a significant difference between TSB-grown BL 137a cells and medium. The comparison of the influence of LB and TSB (both for

cells- and medium-treatment) showed no significant difference among the respective bacteria lines, however, BL 141 TSB medium treatment gained significantly higher amounts of bergenin than the other bacterial lines.

For the second *B. pacumbis* cell line, BP 179, the first set of data shows again the amounts of bergenin produced after a treatment with resuspended autoclaved bacteria cells, on the one hand, and sterile-filtrated LB or TSB medium of the respective bacterial lines, on the other hand (fig. 28 – 31).

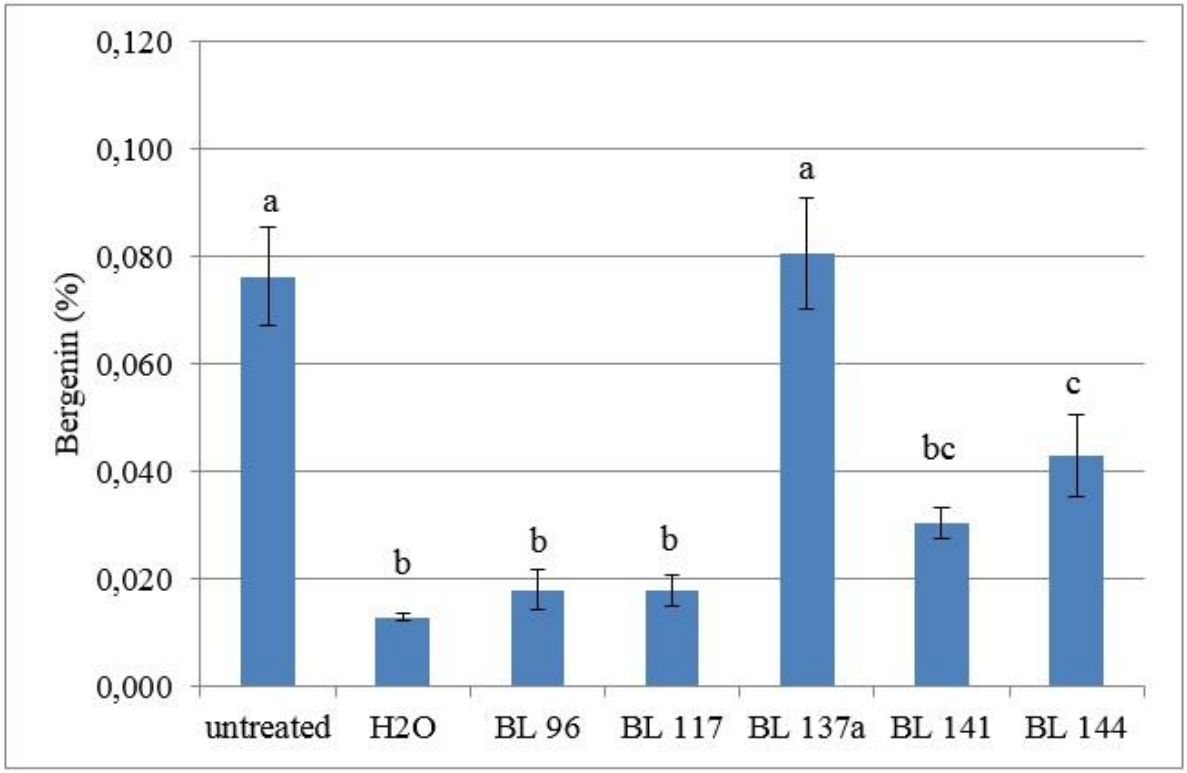

Figure 28. Amount of bergenin in line BP 179 elicited with bacterial cells cultivated in LB medium, compared to untreated cultures and water-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

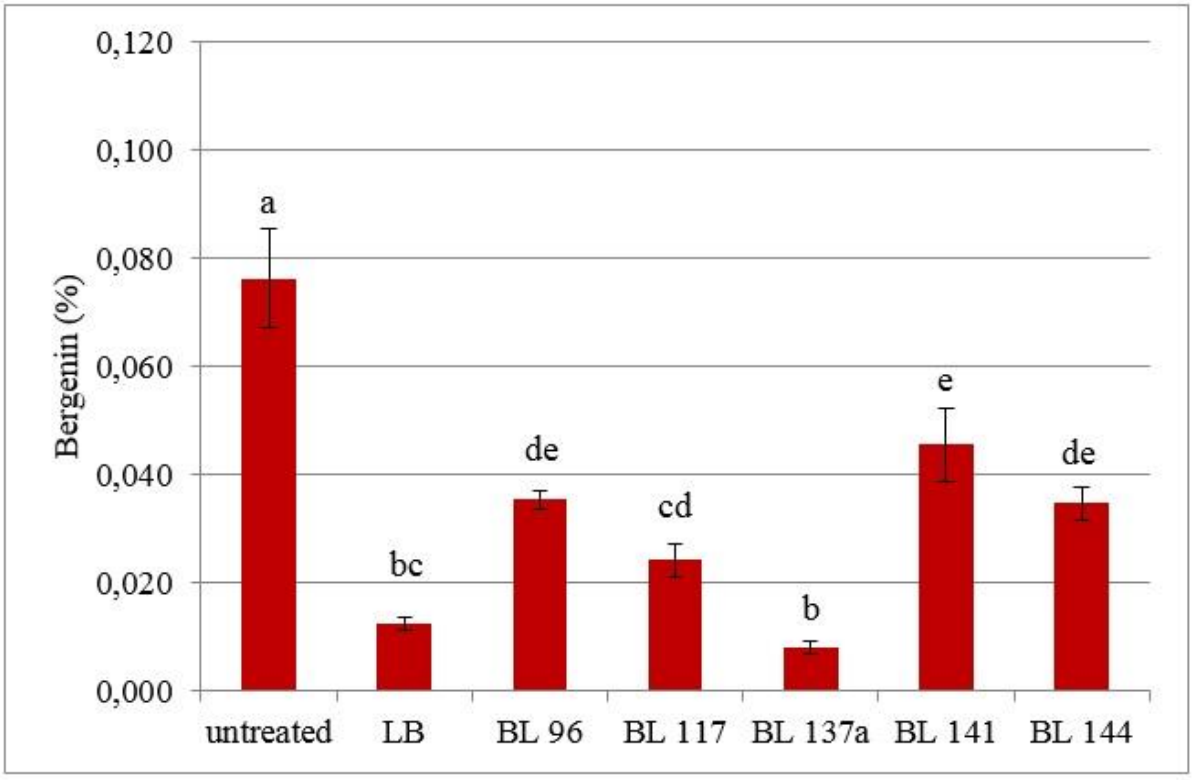

Figure 29. Amount of bergenin in line BP 179 elicited with sterile-filtrated LB medium of the respective bacteria, compared to untreated cultures and LB-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test). Values for BL 137a were below the LOQ.

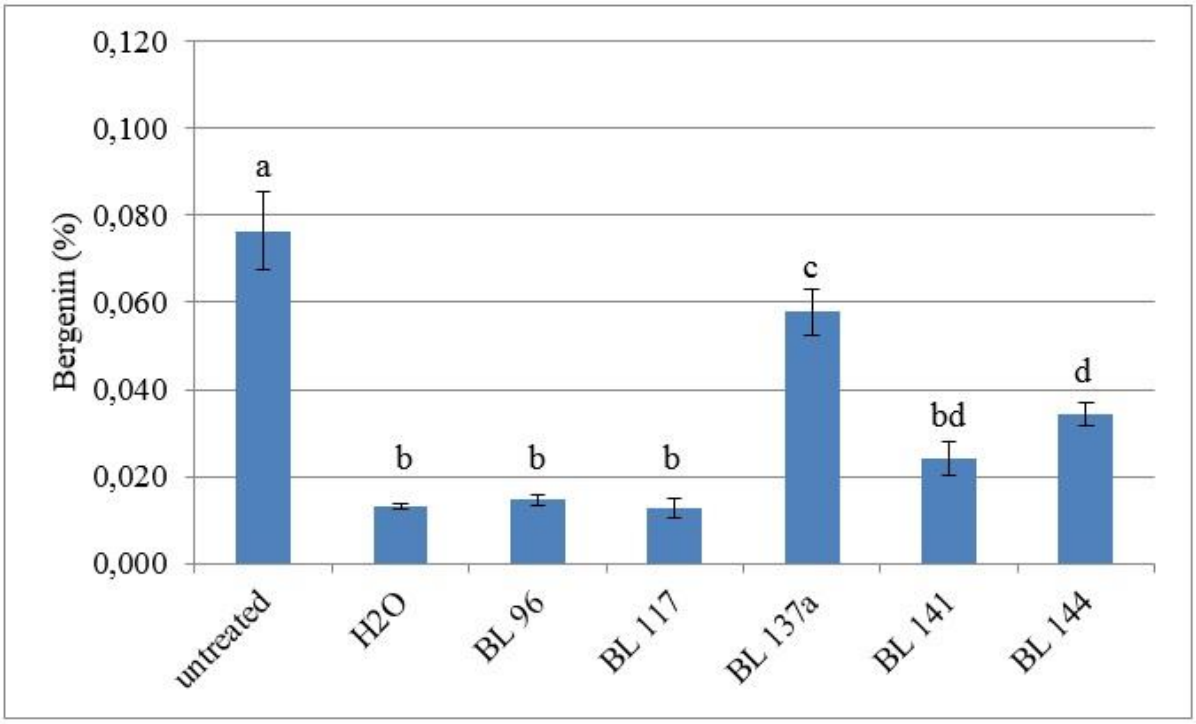

Figure 30. Amount of bergenin in line BP 179 elicited with bacterial cells cultivated in TSB medium, compared to untreated cultures and water-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

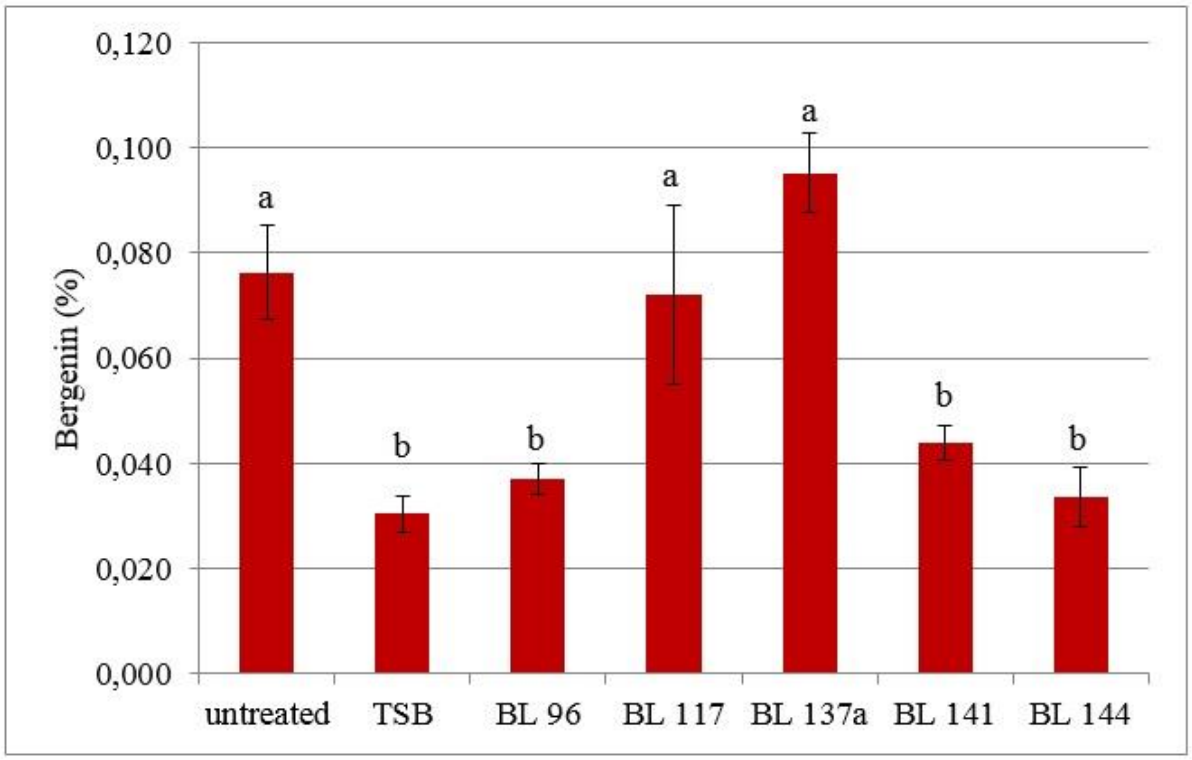

Figure 31. Amount of bergenin in line BP 179 elicited with sterile-filtrated TSB medium of the respective bacteria, compared to untreated cultures and TSB-only treatment. Same letters indicate no statistically significant difference (n=3; one-way ANOVA, Duncan's Multiple Range Test).

The next set of data (fig. 32 – 35) shows again, on the one hand, the different influence of treatment with bacterial cells compared to sterile-filtrated medium (for both LB and TSB medium) and, on the other hand, the influence of the two media (shown for both cell- and medium-treatment).

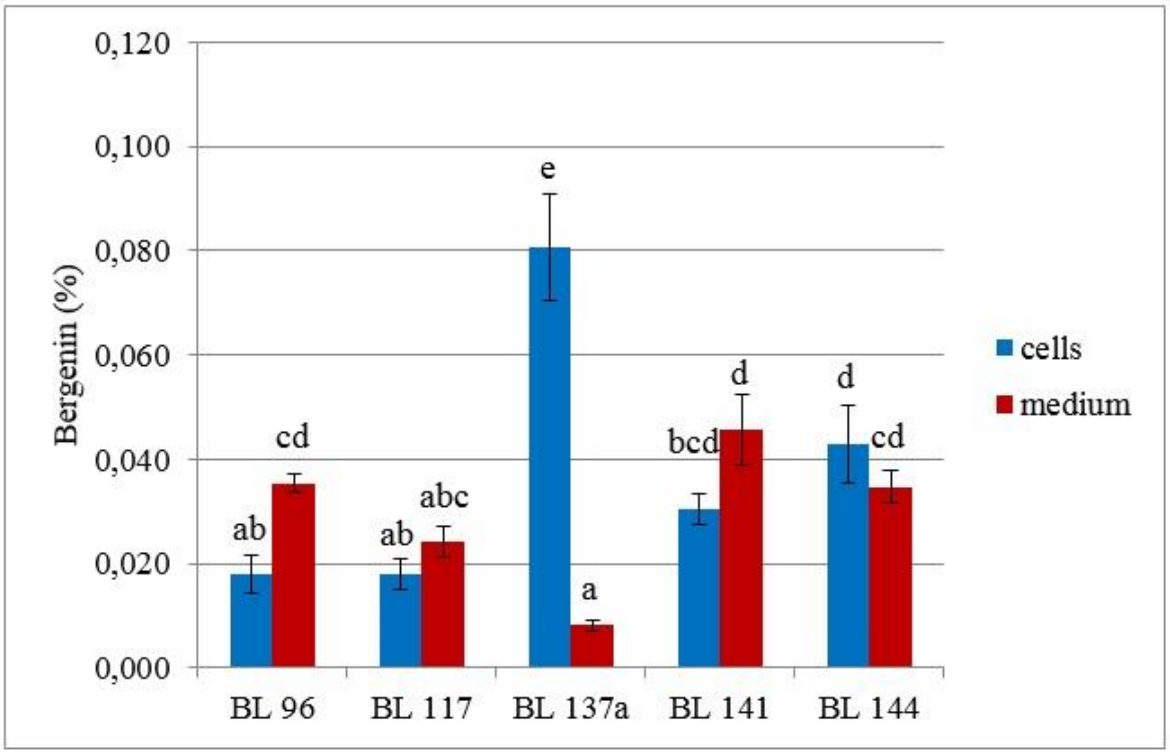

Figure 32. Amount of bergenin in line BP 179 elicited with either autoclaved bacterial cells grown in LB medium, or sterile-filtrated LB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

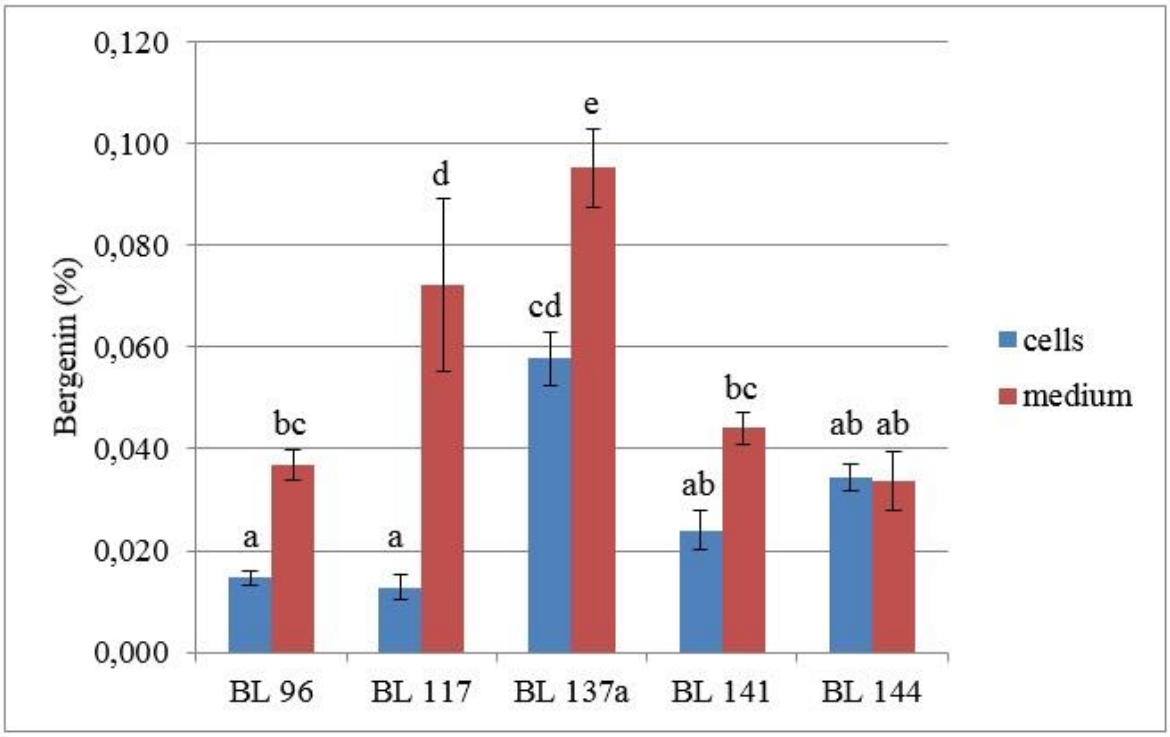

Figure 33. Amount of bergenin in line BP 179 elicited with either autoclaved bacterial cells grown in TSB medium, or sterile-filtrated TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

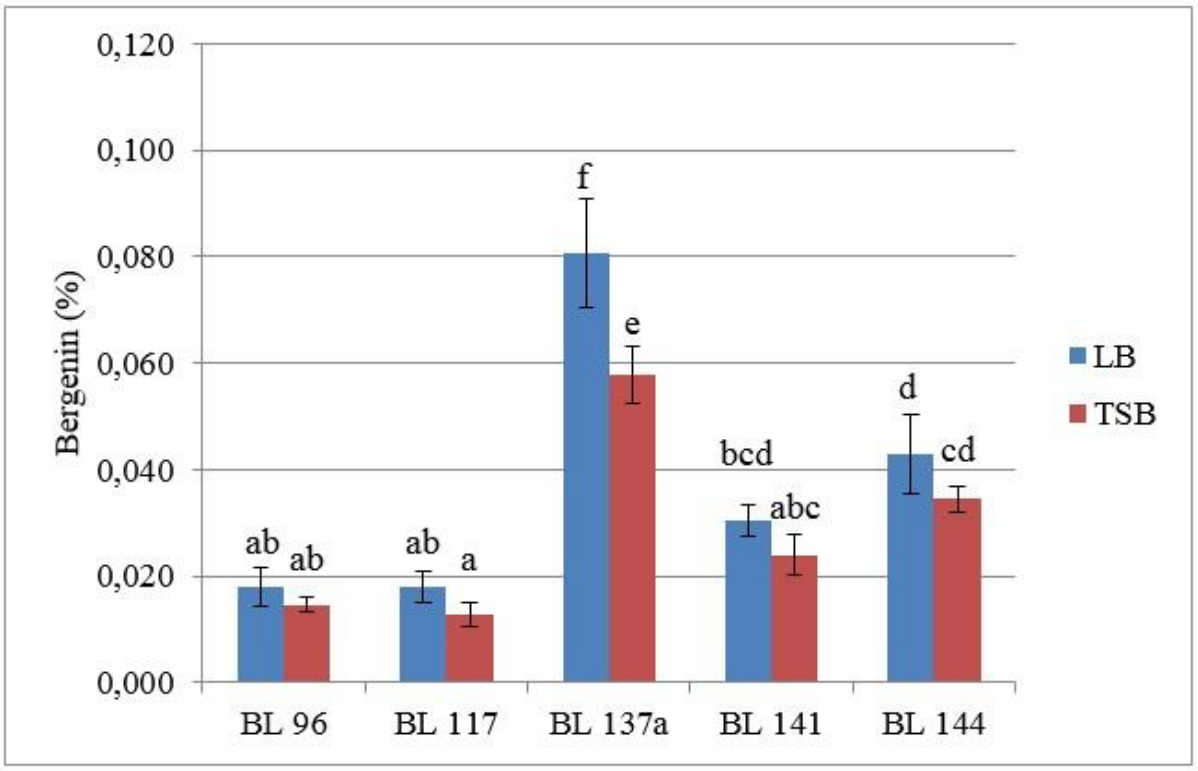

Figure 34. Amount of bergenin in line BP 179 elicited with autoclaved bacterial cells grown in either LB or TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

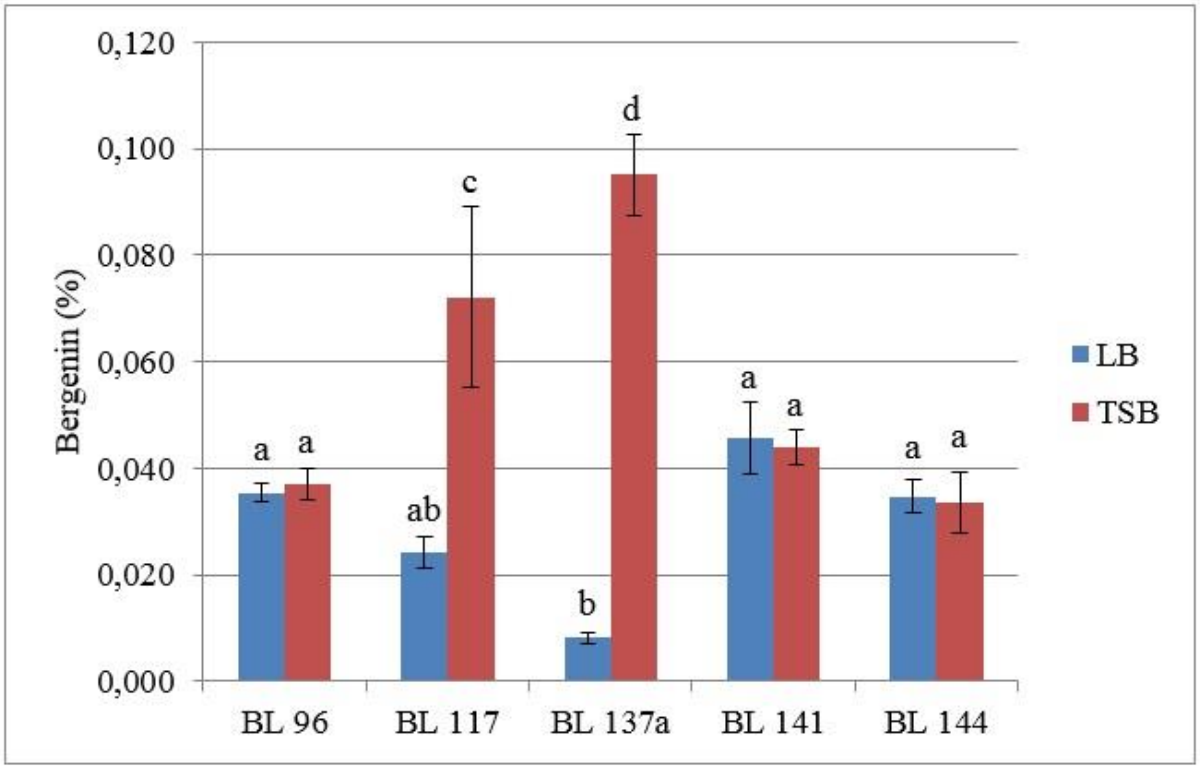

Figure 35. Amount of bergenin in line BP 179 elicited with sterile-filtrated bacterial LB or TSB medium. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

To summarize, most elicitor treatments resulted in decreased bergenin content of cell line BP 179. Only the amounts gained by elicitation with BL 137a cells (grown in LB medium) were not significantly lower than in the untreated cultures; elicitation with BL 144 cells led to significantly higher bergenin content than water-only treatment. Interestingly, the sole treatment with water also led to a statistically significant decrease in bergenin amounts. The elicitation with bacterial LB medium led in all cases to significantly lower bergenin concentrations than in the untreated cultures; treatment with medium of bacterial strains BL 96, 141 and 144 resulted in significantly higher bergenin contents than the control treatment with LB medium. Furthermore, it is noteworthy that the bergenin concentration measured after elicitation with BL 137a medium was below the LOQ of the HPLC method. The treatment with cells grown in TSB medium led in all cases to significantly lower amounts of bergenin than in the untreated cultures; with BL 137a cells this effect was significantly less pronounced than with the other treatments. In cultures treated with TSB medium all but the bergenin amounts measured for BL 117 and 137a were significantly lower than in the untreated cultures; bergenin contents after elicitation with the latter were not significantly different from the untreated control, but were significantly higher than in the other treated cultures. The comparison of cell- and medium-treated cultures (fig. 32) showed the following interesting results: BL 137a cells, grown in LB medium, induced significantly higher bergenin amounts than the other treatments, these were approximately 10 times as high as upon treatment with the corresponding medium (which was however below the LOQ). Furthermore, treatment with BL 96 medium led to significantly higher bergenin production than treatment with the corresponding cells. On the other hand, BL 137a TSB medium was superior to all other treatments (fig. 33); furthermore, the treatment with bacterial medium was also superior to the respective cell-treatment for strains BL 96 and BL 117. In general, the treatments with bacterial TSB medium were (except for BL 144) more effective than the treatment with cells, which can most likely be linked to the medium contents. The comparison of LB and TSB medium (fig. 34) showed that the treatment with BL 137a cells led to significantly more amounts of bergenin than the other bacterial cell lines, the treatment with BL 137a cells grown in LB medium was furthermore significantly more effective than with those grown in TSB medium. Elicitation with bacterial media (fig. 35) led to the significantly highest bergenin amounts with BL 137a TSB medium (which was approximately 11 times higher than with the respective LB treatment). The treatment with BL 117 TSB

medium also led to significantly higher bergenin amounts than the other treaments, and was approximately 3 times as effective as the respective LB medium. Bergenin concentrations after elicitation with the other media did not differ significantly from each other.

The final set of data shows the results depicted as productivity (mg bergenin/l, fig. 36-39).

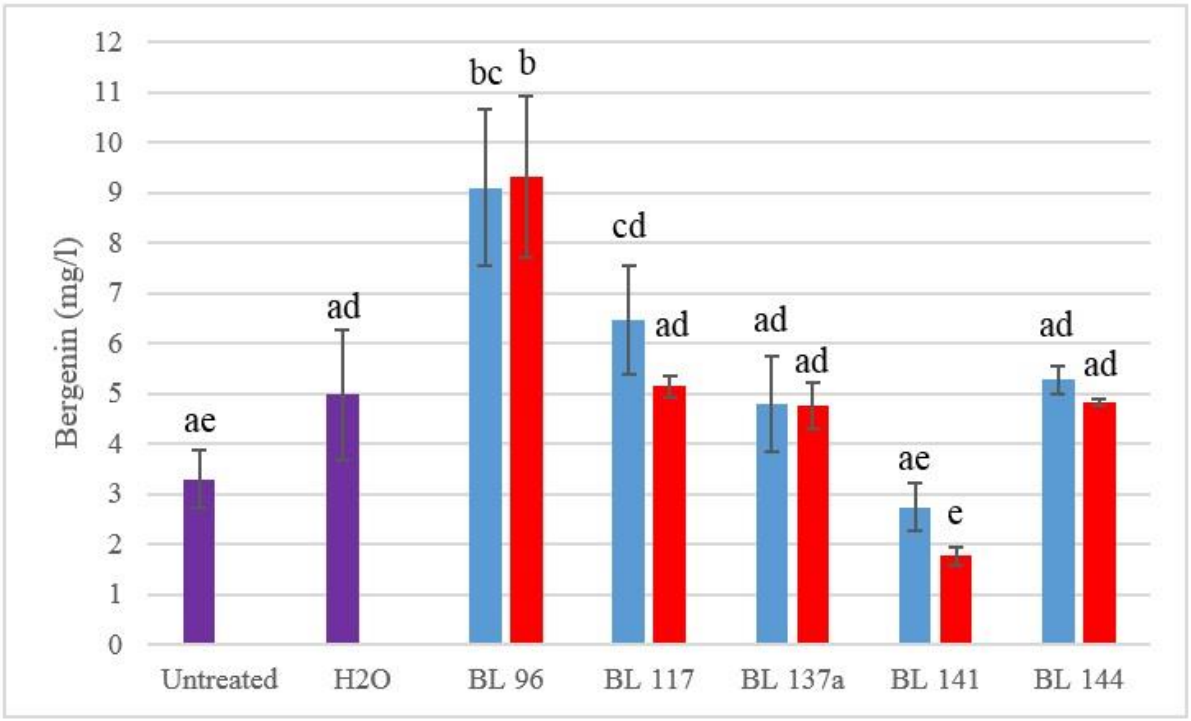

Figure 36. Bergenin productivity of line BP 57 elicited with autoclaved bacterial cells, cultivated in LB (blue) or TSB medium (red), compared to untreated cultures and water-only treatment (purple). Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

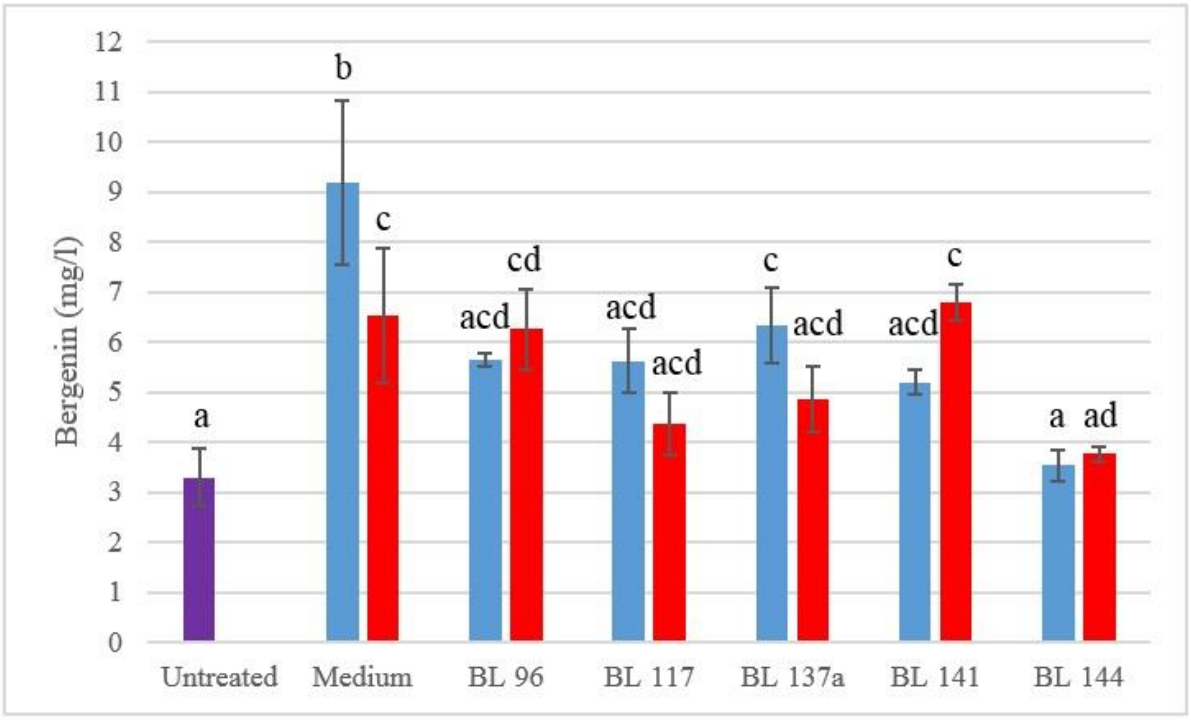

Figure 37. Bergenin productivity of line BP 57 elicited with sterile-filtrated bacterial medium – LB (blue) or TSB (red) – compared to untreated cultures (purple) and medium-only treatment. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

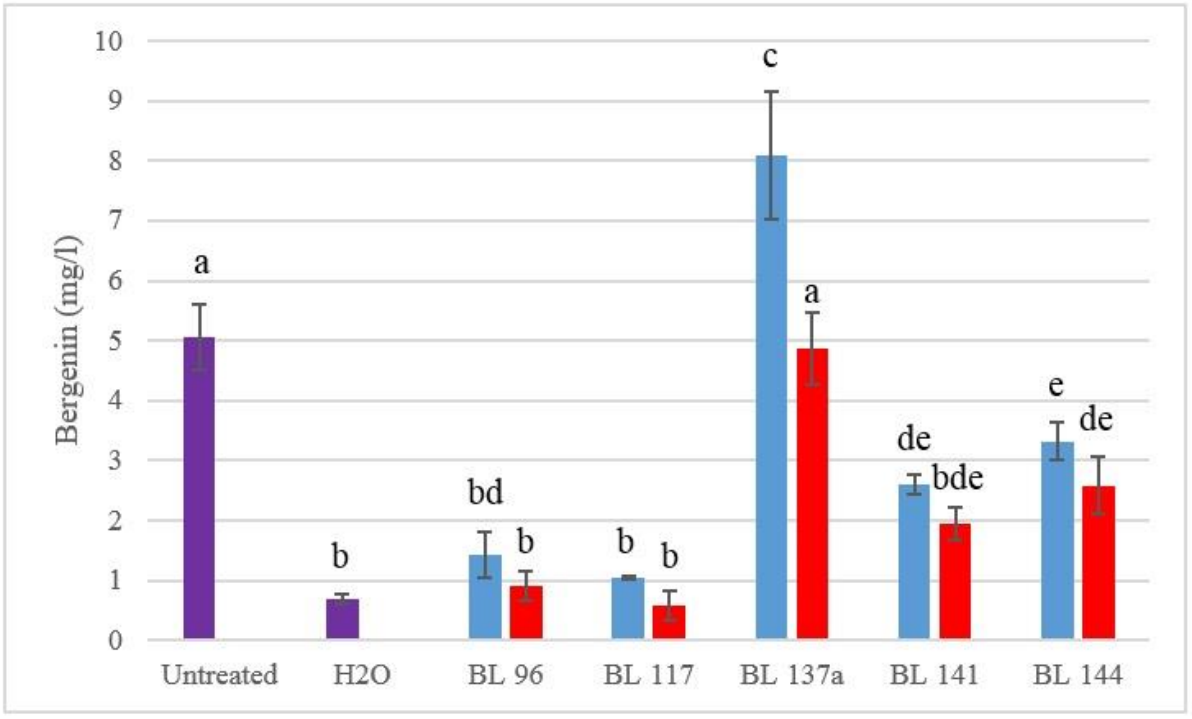

Figure 38. Bergenin productivity of line BP 179 elicited with autoclaved bacterial cells, cultivated in LB (blue) or TSB medium (red), compared to untreated cultures and water-only treatment (purple). Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

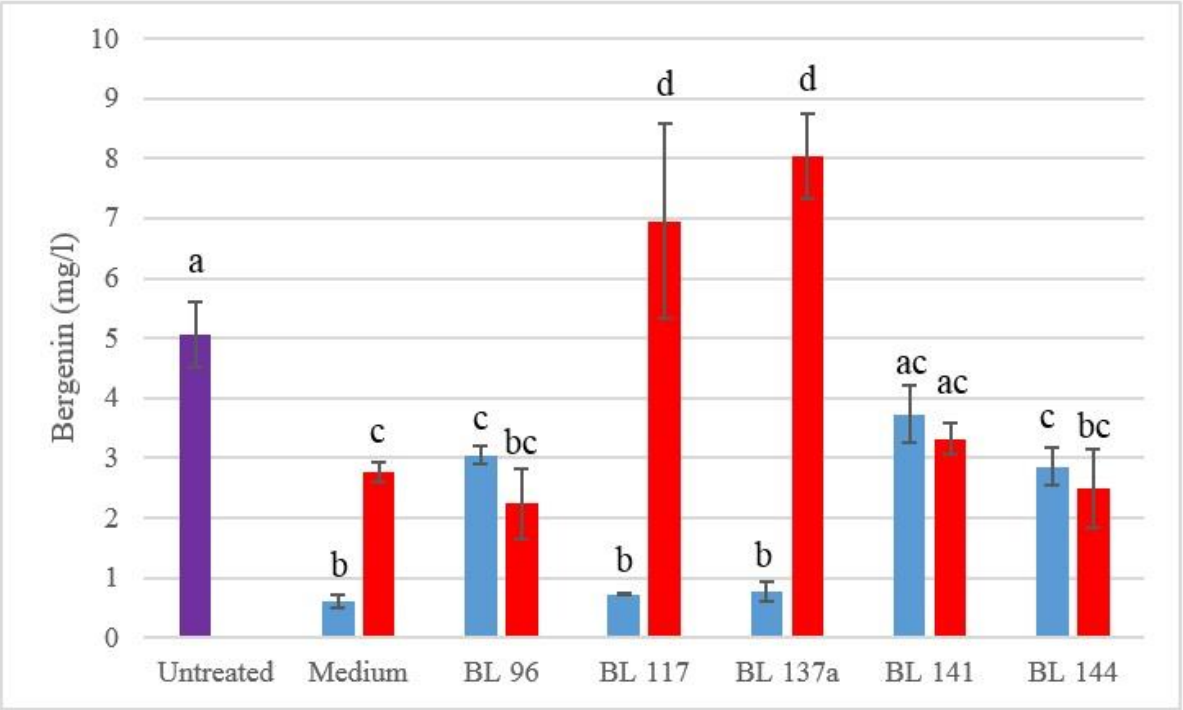

Figure 39. Bergenin productivity of line BP 179 lines elicited with sterile-filtrated bacterial medium – LB (blue) or TSB (red) – compared to untreated cultures (purple) and medium-only treatment. Same letters indicate no statistically significant difference (n=3; two-way ANOVA, Duncan's Multiple Range Test).

The overall tendency remains the same in this depiction showing the productivity of the cultures, however, some interesting points have to be mentioned. In *B. pacumbis* cell line BP 57 the treatment with bacterial media led to a statistically significant increase of bergenin (compared to untreated cells) in medium-only treatments. Furthermore, the treatment with LB medium also resulted in significantly higher amounts of bergenin when compared to the treatments with bacterial media. In cell line BP 179 the treatment with BL 137a cells, both grown in LB and TSB medium, did not significantly inhibit bergenin formation (when the bergenin content per gram of cells is evaluated [fig. 28 & 30], only bacterial cells grown in LB medium did not inhibit bergenin synthesis). Furthermore, the treatment with BL 137a cells, grown in LB medium, showed a statistically significant increase of bergenin (compared to untreated), which was not the case before. The treatment with water still leads to statistically significant lower amounts of bergenin. Both the treatment with BL 117 and BL 137a TSB media now led to a statistically significant increase (compared to the untreated cultures), which is especially interesting as the corresponding LB media both showed a statistically significant decrease (and only led to approximately 1/10 of the bergenin amounts gained with the TSB-counterparts).

6. Discussion

The present thesis aimed at the establishment of cell suspension cultures from callus of *Bergenia pacumbis*, the identification of some previously isolated endophytic bacteria through 16S rDNA sequencing, and elicitation of *B. pacumbis* suspension cultures with preparations of endophytic bacteria.

The callus cultures of *Bergenia pacumbis* which were available for the experiments exhibited a tendency to formation of large cell aggregates. Attempts to obtain fine (i.e. single cell) suspensions only eventuated in a very low viability of the resulting cultures. Further alteration of media consituents (including types and concentrations of plant growth regulators, which has been done previously for the cultures on semi-solid media – cf. Höninger, 2020) could lead to more promising results. The blending method, i.e. cutting up of callus aggregates in liquid medium at high speed, would generally be a very quick and promising approach, especially for slowly growing cultures. However, the sterility of the used device has to be ensured, since in the experiments the occurrence of microbial contamination was the only real disadvantage of the method.

Regarding the results of the phylogenetic characterization of the bacterial endophytes it is noteworthy, that the diversity was (comparably) lower, however, the overall result (sc. a majority of gram-negative bacteria) was consistent with the preceeding findings of Höninger (2020). An interesting detail is the finding that *Prolinoborus fasciculus* shows a very high genetic similarity to *Acinetobacter lwoffii* and was therefore classified as Gammaproteobacterium (fig. 14; Glaeser et al., 2020), in contrast to the previous assumption that it is a member of the Neisseriaceae family and therefore a Betaproteobacterium. For a complete result of the cultivable bacterial endophytes colonizing *B. pacumbis*, it is on the one hand necessary to finish the identification of the remaining samples, on the other hand, already existing data need to be compiled.

The HPLC measurements of the secondary metabolites arbutin and bergenin, the main compounds of *B. pacumbis*, after elicitation of suspension cultures, showed some noteworthy results. There were no detectable amounts of either substance in the nutrient media. This was in general not surprising since it is well known that secretion of secondary metabolites into the medium is often lacking in plant tissue culture (specifically: cell suspension cultures). However, since during the cultivation experiments not only cells

turned dark brown, but also the nutrient medium, and since brown colouring is associated with phenolic compounds (Khosroushahi et al., 2011; Gao et al., 2020), it is to expect that some sorts of phenolic compounds were indeed released into the medium. To elucitate if, and which, compounds were actually responsible for the colouring, in-depth analyses would be necessary. The current HPLC analyses of both cells and media extracts often showed peaks with a retention time in the region of arbutin, this would be a first indication for the presence of gallic acid (Boros et al., 2014).

The lack of arbutin was somewhat surprising, since the used callus had been established from leaf-explants, which are known to usually contain high amounts of arbutin (Höninger, 2020). However, these results are in accordance to the findings of Höninger, who was also not able to detect arbutin in the callus lines.

In regards to the contents of bergenin, there were a couple of interesting observations made. The general amounts which were measured differed from the results of Höninger (2020), who reported 0.03 % for line BP 179 (“BP 2 petiole”) and 0.08 % for line BP 57 (“BP 2 midrib”). In the unelicited BP 179 suspension culture 0.0763 % bergenin could be detected, whereas the reduced amounts of the compound in most of the elicited cultures were comparable to the measurements of Höninger. Line BP 57 on the other hand showed lower contents in the unelicited callus (0.0362 %), in the elicited cultures higher (after elicitation with BL 96 LB cells), equal (after elicitation with BL 141 LB medium) and even lower (after elicitation with BL 117 TSB medium) contents than Höninger could be measured. A lower bergenin content of the unelicited cultures is not surprising, especially considering the rather long subculturing period (Fu et al., 2012; Sanchez-Muñoz et al., 2019), but the higher contents are on the other hand unexpected. This leads to the evaluation of the gained results: as shown above, the results obtained with line BP 57 were in general what would be expected after treatments with elicitors (sc. an increase of desired secondary metabolites). Interesting are especially the bergenin amounts upon treatment with BL 96 cells (amounts approximately doubled, when grown in TSB medium, or tripled, when grown in LB medium, compared to unelicited cultures) and BL 141 media (amounts approximately doubled, in case of LB, or tripled, in case of TSB), compared to the unelicited cultures.

The experiments with line BP 179 show two noteworthy aspects: firstly, all treatments (except for BL 137a and BL 117 TSB medium) caused a significant decrease in bergenin production compared to untreated cultures, which in itself is rather uncommon

(Wang et al., 2012; Gonda et al., 2013; Yan et al., 2014). Secondly, a clear dichotomy between the two cell lines was evident. The adverse effects of elicitation on bergenin formation were not the only difference between the cell lines: as mentioned before, line BP 179 had a rather friable texture. Furthermore, even when a pronounced brown colouring of the callus occurred (which is in general associated with cell death), the cells still showed a high amount of viability when investigated via FDA. This stands in contrast to line BP 57 which behaved 'normally' (i.e. consisted of only a small percentage of viable cells). Differences were also detected in fresh and dry weight: 48 hours after elicitor treatment, both fresh and dry weight of the BP 179 cells on average were lower, and while the mean dry weight of BP 179 was 6.6 % of fresh weight, for BP 57 it was 4.8 %. There was also a difference between the lyophilized callus cells: in case of BP 179, they had a paper-like consistency (i.e. friable and light) and were of generally white (after elicitation with cells) or grey/blackish (after elicitation with medium) colour. Lyophilized cells of line BP 57 were small, rather hard and heavy, and either white – after elicitation with cells – or brown – after elicitation with medium.

All these morphological characteristics could indicate some sort of (epi-)genetic difference, even though they were both derived from the same plant and organ. Similar findings have also been described e.g. for ginseng by Reshetnyak et al. (2008) or for tea (Yang et al., 2012). This makes it likeable, that these or other genetic deviations were responsible for the contrastive results after elicitation. If the assumption, that the production of bergenin is correlated with the amounts of H_2O_2 produced (and subsequently increased amounts of phytohormones; see chapter 2.4.) is correct, this could indicate either a different reaction of stress-involved enzymes (superoxide dismutase / catalase etc.) towards the treatments (Ma et al., 2019; Sadeghi et al., 2020) or even a different general activity of these enzymes.

Another possible explanation (though rather speculative) is a biphasic/bilateral reaction towards endophytic elicitors – dependent on the situation – which is in this case characterized by the initially high amounts of secondary metabolites in line BP 179 (due to a stress reaction), wherefore the reaction towards the endophytes is characterized by stress-reduction (which leads to a decrease of, in this case, bergenin). On the other hand, low contents of secondary metabolites (and stress) in line BP 57 allow for a priming-like reaction with an increase of stress and simultaneously also an increase in bergenin. The reason why

line BP 179 experiences stress under the same conditions as line BP 57 can be seen in the genetic difference (which exactly is unclear) and can probably be linked to the temperature: during cultivation experiments it was shown, that cultures, which had to be subcultured the following week, grown at the normal cultivation temperature of 25 °C showed a stronger discolouring (browning) compared to cultures grown at approximately 21 °C – due to an outage of the temperature regulation over the weekend. Höninger (2020) also compared callus cultures in regards to their response to different temperatures and noticed a similar effect when comparing cultures grown at 25 °C to those grown at 28 °C.

The assumption of a metabolic shift, i.e. a decrease of bergenin in favor of one or more putative other secondary metabolites (Rasmussen et al., 2008; Scherling et al., 2009), might also be possible and would imply that the reaction is not necessarily linked to a specific genetic or enzymatic mutation, but can be seen as the reaction towards different endophyte species (cf. Groten & Barz, 2000 – who performed their experiments with pathogens, however). All the above mentioned theories, however, do not provide an explanation for the pronounced decrease of bergenin content in line BP 179 after water-only treatment.

Further investigations are necessary for the elucidation of the underlying mechanisms of this dichotomy in the two studied cell lines of *Bergenia pacumbis*. Other possible research could deal with additional callus lines and/or callus obtained from other plant tissues than leaves. Elicitation with other endophytes as well as common abiotic elicitors would provide new data for the comparison with the already gained results. Lastly, the establishment of co-cultures with the most promising endophytic elicitors could additionally provide information about the long-term effects on the production of bergenin and other secondary metabolites.

7. References

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