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„The impact of endophytes on the alkaloid pattern of cell suspension cultures of *Atropa belladonna* L.“

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List of contents

Abbreviations

AB	*Atropa belladonna* L.
DW	Dry weight
FW	Fresh weight
HPLC	High Performance Liquid Chromatography
Kin	Kinetin
MS	Murashige & Skoog medium
NAA	1-Naphthaleneacetic acid
rpm	Revolutions per minute
SM	Secondary metabolites
P	Pellet
S	Supernatant

1. Abstract

Plant cell suspension cultures typically produce only very low amounts of secondary metabolites. This is generally ascribed to the lack of differentiation, but the absence of endophytes may also be a cause. The present study deals with the impact of endophytes of *Atropa belladonna* L. on the formation of tropane alkaloids in suspension cultures of the plant. For this purpose, cell cultures were established from belladonna callus lines. In appropriate medium, they were grown and analyzed for their tropane alkaloid content. Cell lines, which showed significant production of tropane alkaloids were multiplied by several sub-culturing steps and characterized for growth. 7-day-old cell suspension cultures in the exponential phase were then elicited with autoclaved preparations of 4 different strains of endophytes. For this purpose, endophyte cultures were centrifuged and "pellet" and "supernatant" elicitor preparations were obtained.

HPLC analyses showed that the atropine content in suspension cultures treated with pellet elicitors decreased significantly in comparison with untreated controls. After elicitation with supernatant preparations, the atropine amount decreased significantly, too. In comparison with the controls, the scopolamine amount in cell suspension cultures of *Atropa belladonna* showed a significant increase after treatment with pellet elicitors. The most pronounced effect on the scopolamine content of in vitro cultures was achieved through treating them with supernatant elicitors. Compared to the control, every sample showed a significant increase of the scopolamine amount. A significant rise of scopolamine was also reached by the supernatant of one specific bacterial strain, when compared to the TSB-control.

Zusammenfassung

Pflanzliche Zellsuspensionskulturen produzieren typischerweise nur sehr geringe Mengen an sekundären Metaboliten. Dies wird im Allgemeinen auf die fehlende Differenzierung zurückgeführt, aber auch das Fehlen von Endophyten kann eine Ursache dafür sein. Die vorliegende Arbeit beschäftigt sich mit dem Einfluss von Endophyten von *Atropa belladonna* L. auf die Bildung von Tropanalkaloiden in Suspensionskulturen der ebengenannten Pflanze. Zu diesem Zweck wurden Zellkulturen aus Belladonna-Kalluslinien etabliert. In einem geeigneten Medium wurden sie gezüchtet und auf ihren Tropanalkaloidgehalt getestet.

Zelllinien, die eine hohe Produktion an Tropanalkaloiden aufwiesen, wurden durch mehrere Subkultivierungsschritte vermehrt und mit Hilfe einer Wachstumskurve charakterisiert. Anschließend wurden 7 Tage alte Zellsuspensionslinien, die sich in der exponentiellen Phase befanden, mit autoklavierten Zubereitungen von 4 verschiedenen Endophytenstämmen. Zu diesem Zweck wurden Endophytenkulturen zentrifugiert, um so "Pellet"- und "Überstand"-Elizitorzubereitungen zu erhalten.

Die HPLC-Analysen zeigten, dass der Atropin-Gehalt in Suspensionskulturen, welche mit Pellet-Elizitoren behandelt wurden, im Vergleich zu den Kontrollen signifikant abnahm. Auch nach der Behandlung mit Überstand-Elizitoren sank die Atropinkonzentration im Vergleich zu den Kontrollen deutlich. Der Gehalt an Scopolamin in Zellsuspensionskulturen von *Atropa belladonna* wies nach Behandlung mit Pellet-Elizitoren eine signifikante Zunahme auf. Die stärksten Auswirkungen auf den Scopolamingehalt in *in vitro* Kulturen wurden durch Behandlung mit Überstand-Elizitoren erzielt. Im Vergleich zur Kontrolle zeigte jede Probe einen signifikanten Anstieg der Scopolaminmenge. Eine beträchtliche Erhöhung des Konzentration dieses Alkaloids wurde auch durch den Überstand eines spezifischen Bakterienstamms im Vergleich zur TSB-Kontrolle erreicht.

2. Introduction

2.1. *Atropa belladonna* L. and tropane alkaloids

The family Solanaceae contains many genera well-known and widely utilized by human society, e.g. tomatoes, potatoes, or tobacco. All these plants are widely spread all over the world. One of these is the perennial herbaceous plant *Atropa belladonna* L. (Figure 1), also termed as „deadly night shade" or "belladonna", which is endemic to Western-Asia, Europe and North- Africa. The name *A. belladonna* derives from Atropos, goddess in Greek mythology, who chose the way of death for mortals, and from the Italian word "belladonna", which means beautiful woman. Linné probably decided on this name as he wanted to hint at the plants' toxicity (Al-Ashaal et al., 2013; Goldberg, 2009; Hänsel and Sticher, 2010; Lee, 2007; Spiegl, 1996).

Figure 1: The plant *Atropa belladonna* (https://de.wikipedia.org/wiki/Schwarze_Tollkirsche#/media/Datei:Illustration_Atropa_bella-donna0_clean.jpg, retrieved on September 10, 2019).

The plant's macroscopic and microscopic characteristics are described by Rahfeld (2011). This perennial, herbaceous plant has green to brown-green colored leaves, which are often rolled-up and tangled when dried. The petiolate leaves are 5 to 25 cm long and their width

can be 2.5 to 12 cm. The blossom has connate sepals and the crown is bell-shaped. The green to violet-black colored fruits, which are spherical-shaped and taste slightly bitter, turn up in Europe during the summer months. A very large amount of brown-colored seeds are produced by them. The leaves' bifacial profile shows a single-rowed palisade mesophyll, a sponge-tissue and particles of calcium oxalate, which is located in idioblasts. Glandular hairs situated on the surface of leaves are single-rowed, long and have a multicellular stem.

Within the family of Solanaceae the species *A. belladonna* is most noteworthy in the production of tropane alkaloids (Yang et al., 2011). These alkaloids (Figure 2) are used as medicines since ancient times by mankind. Notes of their applications in folk medicine of several ethnic groups are plenty. Ethnopharmacologically, concoctions of the plants were employed as poison and philter of different kinds, as painkiller and hallucinogen (Grynkiewicz and Gadzikowska, 2008; Henry, 1949; Leake and Pelikan, 1976).
As *A. belladonna* leads to dilation of eyes' pupils, women in Ancient Greece often used the plant to achieve a more attractive appearance. In the Middle Ages witches, sorcerers and professional poisoners also made use of the plant's extracts. Ingesting these brews gave a sensation of flying. This might explain the connotation to witches (Hofmann and Schultes, 1987; Lee, 2007; Tombs and Silverman, 2004).

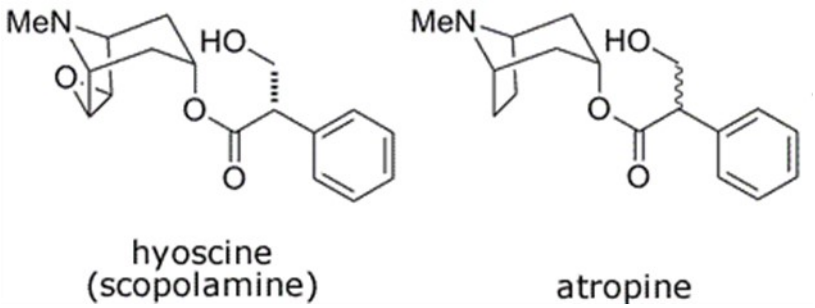

Figure 2: Scaffolds of the tropane alkaloids atropine and scopolamine (https://www.bing.com/images/search?view=detailV2&ccid=Qw%2fTtheZ&id=4D15808E2FFE56D46C7A78D18410772124E282D6&thid=, retrieved on September 10, 2019).

According to Mothes et al. (1985), alkaloids of *A. belladonna* are produced in the root system of the plant, from where they are translocated to other plant tissues (Zayed and Wink, 2004).

In reference to dry weight, the alkaloid concentration can be 0.45-0.85 % in roots and 0.2-1.5 % in leaves. The major alkaloid is S-(-)-hyoscyamine, which racemizes to atropine while drying. Leaves also contain S-(-)-scopolamine in small quantities. This compound shows only low tendency to racemize to the metabolite atroscine. The production of (S)-hyoscyamine is twenty times higher than that of (S)-scopolamine, hence the ratio of these tropane alkaloids in plant is usually 20:1 (Hänsel and Sticher, 2010). As analyzed by Sporer et al. (1992) there are diurnal fluctuations of the alkaloid content in the plant.

Great endeavors to isolate the pure active principles of tropane alkaloids in the 19th century were followed by their structural elucidation and chemical synthesis in the 20th century (Christen, 2000; Fodor, 1971).

Due to progress of modern medicine these days, there is a great commercial demand for tropane alkaloids, which are produced from transgenic medicinal plants. In the last decades, great development in plant biotechnology opened up new possibilities for the efficient production of plant derived drugs (Parr, 1989; Zhang et al., 2007).

All secondary metabolites (SM) derived from the plant *A. belladonna* have a tropane scaffold in common and are separated in tropine and ecgonine derivatives. Atropine and hyoscine belong to the tropine alkaloids. (S)-Hyoscyamine, the principal alkaloid of *A. belladonna*, is an ester of tropine with (S)- or (R)-tropic acid. The tropic acid might be formed by a rearrangement of scaffold of phenyl lactic acid originated from L-phenylalanine (Grynkiewicz and Gadzikowska, 2008).

(+)-Hyoscyamine and (-)-hyoscyamine distinguish themselves substantially in their biological activities. According to many studies, the biological potency of S-(-)-hyoscyamine is 30 fold greater than that of R-(-)-hyoscyamine (Leffingwell, 2003).

In medicine, the racemate atropine is used (Gyermek, 1997). Compared to the enantiomers, atropine is more robust, less susceptible to enzymatic destruction and can be normalized for potency.

Anisodamine, also known as 6-β-hydroxytropine, is formed by the action of the enzyme 6-(R)-hydroxylase (H6H; hyoscyamine 6β-hydroxylase). It is a precursor of scopine, which represents a partial structure of scopolamine, the second main alkaloid of *A. belladonna* (Grynkiewicz and Gadzikowska, 2008).

The effects of tropane alkaloids include the blocking of postganglionic muscarinic acetylcholine receptors, whereby atropine leads to greater inhibition than the second major alkaloid scopolamine (Leffingwell, 2003).
In low doses atropine has only minor impact on the central nervous system, but higher doses cause clouded awareness and confusion.
Scopolamine has effects on the central nervous system as well. In low doses, the drug leads to sedation and in greater doses it provokes hallucinations and disorientations. (Grynkiewicz and Gadzikowska, 2008).
Tropane alkaloids are antagonists of the muscarinic receptor of neurotransmitter acetylcholine. This fact explains their anticholinergic effects on different body tissues (Verpoorte and Alfermann, 2013). As they block the muscarinic receptors of acetylcholine and cause a reduction in activity of the parasympathetic nervous system, they are named parasympatholytics (Aktories et al., 2013).

Due to their clinical effects, tropane alkaloids as agents applied in medicine are in great demand (Al-Ashaal et al., 2013). Depending on the dose, atropine causes various effects on different body tissues. Low doses of atropine lead to a decrease of the heart rate and high doses may provoke tachycardia. Owing to these effects, atropine is therapeutically used in patients suffering from cardiovascular disorders, especially from bradycardic arrhythmia and acute myocardial infarction (Das, 1989).
Atropine induces cardiovascular changes and may cause atrioventricular block (Aktories et al., 2013).
Moreover, atropine leads to an inhibition of the secretory activity in the respiratory tract (Centanni et al., 1998) and to a dilation of the bronchial tubes. Therefore, atropine is therapeutically used to treat nonallergic rhinitis to prevent profound mucus production. Bronchial musculature spasms of cholinergic origin can be prevented by atropine. In this case, drugs derived from atropine such as ipratropium and tiotropium are used in combination with β2-adrenoceptor agonists and theophylline.

Besides these effects, atropine has an impact on the smooth muscles. It is able to prevent contraction of smooth muscles, which coat the bile ducts, the gastrointestinal tract and the urinary tract. As many side effects are caused by atropine, a well-tolerated derivate of

scopolamine, namely butyl-scopolamine, is used for spasmolytic therapy (Lüllmann and Mohr, 2006).

The usage of scopolamine as drug is wide-spread. The principal therapeutic application of scopolamine as antiemetic agent is explained by its effect on the vomiting center of the brain. Therefore, postoperative nausea and vomiting can be treated with scopolamine (Antor et al., 2014; Renner et al., 2005). Parkinsonian tremor and premedication for anesthesia belong to the field of therapeutic applications of scopolamine as well (Grynkiewicz and Gadzikowska, 2008; Mirakhur and Dundee, 1979).
As scopolamine has a greater biological activity and shows less negative effects than atropine, it is more frequently used as pharmaceutical drug (Jaremicz et al., 2014; Wang et al., 2011; Xia et al., 2016).

Dilation of the pupils and paralysis of the ciliary muscle in the eye may be evoked by atropine, the main alkaloid of *A. belladonna* (Arnold et al., 2004). Consequently, this compound can be used for diagnostic inspection of the eye in ophthalmology. Atropine causes long-lasting pupil dilation in the human's eye (Salazar and Patil, 1976). Drugs derived from atropine causing a briefer dilation effect in the eye are preferred to hyoscyamine (Aktories et al., 2013).

Intoxication with the plant itself might happen by eating the leaves or the berries, as the tropane alkaloids are located there. First signs of intoxication are manifested after ingestion of 0.5 to 1 mg of atropine. Doses of 100 mg of atropine are life-endangering as cessation of breathing occurs (Aktories et al., 2013; Rajput, 2013).

Intoxications with insecticides or nerve agents such as soman, an anticholinergic compound usually deployed as chemical warfare nerve agent, can lead to death. Acute fatal effects result from respiratory failure. Atropine can be used as an antidote in case of intoxication with such organophosphorus derivatives (McDonough et al., 1989).
Nevertheless, finding the right dose of atropine for poisoned victims is very difficult, as the exact amount of organophosphorus toxin that has been ingested and its concentration in the blood is normally unknown (John et al., 2010).

Even at usual therapeutic doses, individual fluctuations of atropine concentrations in the blood and different reactions to it (toxic and allergic) have been documented. That higher doses of this antidote can lead to dramatic outcomes is conceivable (Robenshtok et al., 2002).

2.2. Production of plant secondary metabolites *in vitro*

Depending on family and species, plants produce numerous compounds of primary metabolism. Sometimes, these are utilized as taxonomic markers in categorizing plants (Bennett and Wallsgrove, 1994; Kabera et al., 2014). Primary metabolites like e.g. carbohydrates, amino acids or lipids are essential for normal growth, evolution and propagation of the plant (Bourgaud et al., 2001). Secondary metabolites (SMs) such as tannins, alkaloids, terpenoids, peptides, and antibiotics are not essential for the plant; however, they have an important ecological function (Kossel, 1891). Kossel was the first scientist who determined a concept for secondary metabolites setting them against the already known primary metabolites of plants (Kossel, 1891). Before he defined a term for secondary metabolites, they were seen as “waste” of primary metabolism.

Due to the advancements in biochemistry and molecular biology, it has been shown that secondary metabolites play an important role in the adaption of plants to their environment. Among others, SMs have antibiotic, antifungal, antiviral and allelopathic effects. Therefore plant SMs play a key role in defending the plant against any harmful external biotic or abiotic stress.

They also have a bearing on the animal world like e.g. on insects, where they serve as attractants for seed distribution (Bourgaud et al., 2001; Kabera et al., 2014).

Some SMs are responsible for the flavor and aroma of fruits and vegetables, and some are beneficial for human health for example as antioxidants, which act as radical quenchers (Cai et al., 2003; Kroymann, 2011; Zheng and Wang, 2001).

Owing to the developments in plant biotechnology during the last decades and the increasing interest in plant-derived drugs, recent research has focused on medicinal plants as potent sources for drugs, food additives and fine chemicals (Zhang et al. 2007; Zhao et al., 2005).

Compared to molecules of synthetic origin, phytochemicals are natural metabolites. Not only their biological activity makes them special, but also the circumstance to be substrates for one or more transporters, which bring the metabolites to the inside of cells, where they carry out their effects. (Harvey et al., 2015).

To obtain a potent system for efficient production of SM under controlled conditions plant cell and tissue cultures were extensively evaluated. Hairy root cultures were often used as organ culture systems for production of desired compounds in many laboratories (Hashimoto et al., 1993; Jung et al., 2003; Zhang et al., 2007). However, only a sparse number of *in vitro* culture systems could be brought to the industrial scale.

There are only a few examples of marketed plant SMs manufactured by plant cell cultures. One of these is the anticancer drug paclitaxel, also known as Taxol®, a diterpenoid with inhibiting effects on the mitosis. The substance is produced by *Taxus* species and is used for anticancer therapy. The first isolation of this drug had been through extraction from the bark of *Taxus brevifolia*. However, the paclitaxel content in *Taxus brevifolia* is very low. Because of the increasing use of paclitaxel in medicine, new sources for this valuable drug had to be found (Ketchum and Gibson, 1996).

Plant cell cultures of *T. brevifolia* were eventually demonstrated to be an alternative source for efficient paclitaxel production (Christen et al., 1991).

Many studies mention the opportunity to produce shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon* at commercial levels (Fujita et al., 1981; Ling et al., 2011; Rao and Ravishankar, 2002). Shikonin has anti-HIV (human immunodeficiency virus) and anti-inflammatory effects (Chen et al., 2003). Another study mentioned its traditional use as anti-cancer agent (Wang et al., 2019).

Another example for the production of SM in plant cell cultures is berberin, a biologically active compound of *Coptis japonica* applied in various fields of medicine. It is used as an antibacterial, antimalarial and stomach agent in Asia and China. The great demand for berberin requires a production on industrial scale. Through an improvement of culture conditions, plant cell cultures can be induced to produce high amounts of berberin (Matsubara et al., 1989; Yamada and Sato, 1981).

A considerable amount of research was done investigating tropane alkaloid production in cell and organ cultures of *Atropa belladonna* and other members of the family Solanaceae (Oksman-Caldentey and Arroo, 2000; Zayed and Wink, 2004).

Compared to field grown plants, one study showed a higher amount of atropine and scopolamine by means of transgenic hairy root cultures of *A. belladonna* (Kamada et al., 1986). Another publication dealt with callus cultures of *A. belladonna*. It showed that levels of SMs in calli deriving from mother plants with great amount of tropane alkaloids had no significant difference compared to those in calli originated from plants with low SM production (Simola et al., 1988). One work investigated callus cell lines containing higher amounts of tropane alkaloids than leaves of the intact mother plant (Khater et al., 2013).

Al-Ashaal et al. (2013) stated that the content of the total tropane alkaloids in two callus cell lines of *A. belladonna* (6.9 mg/g DW & 2.98 mg/g DW) was lower than the content in leaves of the original plant (8.7 mg/g DW).

Since many pharmaceuticals with abundant use in medicine derive from plants (Rao and Ravishankar, 2002; Wink et al., 2005; Newman and Cragg, 2016), the production of desirable SMs in plant cell and organ cultures is of great interest. Under proper conditions *in vitro* cultures could be used to produce SM of standardized quality continuously, without any seasonal limitations, in large bioreactors (Wink et al., 2005).

Furthermore, isolation of the SMs from *in vitro* cultures is easy, fast and cost-effective compared to the extraction of field-grown plants.

Another advantage of the production of SM in *in vitro* cultures is the absence of many other, unwanted metabolites, which only occur in the intact plant (Karuppusamy, 2009). Moreover, the chemical syntheses of some compounds needed in medicine are extremely complex or economically impracticable, thus a plant-based production is necessary (Namdeo, 2007; Yue et al., 2016).

In the intact plant, the SM formation might occur only during a specific growth stage, or with specific requirements related to the time of the year, stress or nutrient availability (Ramirez-Estrada et al., 2016). When a plant producing desired compounds is endangered or cannot be domesticated, plant cell and organ cultures represent a good alternative to wild harvest of plant material (Karuppusamy, 2009).

Another great advantage of using plant cells for the production of medicinal agents is the fact that they do not host any human pathogens or endotoxins (Hellwig et al., 2004).

The cultivation of some plants producing desirable compounds can take a long time. For example, *Panax ginseng* needs to be grown for five to seven years until the roots are ready for harvesting (Bonfill et al., 2002).
Similarly, *Taxus* trees need approximately 60 years to grow to a size at which a reasonable, yet very small amount of paclitaxel could be extracted (Tabata, 2004).
Although many studies have investigated the biotechnological production of desired substances by *in vitro* plant cell and organ cultures in the last years (Bhojwani, 2012; Effert, 2018; Ling et al., 2011; Mulabagal and Tsay, 2004; Ramirez-Estrada et al., 2016; Shuler, 1981; Vanisree et al., 2004), there are still many restrictions in using plant cell cultures. Many attempts to manufacture the desired substance using *in vitro* cultivation of plant cells or tissues have failed.
One reason for these failures might be the lack of knowledge on the complete biosynthetic pathways and mechanisms, which are relevant to the SM formation in plants (Dörnenburg and Knorr, 1995; Namdeo, 2007).This lack of knowledge showed to be the first obstacle in developing industrial prodcution. Problems like aggregate formation, slow growth, a low production of SM, a great susceptibility to diverse forms of stress and genetic instability have been responsible for a decline in the research into the SM production in plant cell cultures (Dörnenburg and Knorr, 1995).
Some reasons for the low yields of SMs produced by plant cell cultures are described by Smetanska (2008). Plant cell cultures lack differentiated cells and therefore specialized structures such as oil glands, glandular trichomes or glandular epidermis are absent. In the intact plant, the accumulation of secondary metabolites occurs in compartments where they cannot harm other plant tissues. In plant cell cultures there is no such possibility of compartmentalized accumulation of SM, and probably therefore they are produced only at low levels (Isah et al., 2018).

Plant organs are composed of totipotent, differentiated cells. “Totipotent” implies that every single plant cell is capable of giving rise to any cell type or even a complete plant (Mustafa et al., 2011; Steward et al., 1958). The term “differentiated” means that the plant is composed

of specialized types of cells, arising from undifferentiated (stem) cells (Lyndon, 1990). These various types of differentiated cells in plants form several kinds of tissues such as parenchyma tissue, collenchyma tissue, and sclerenchyma tissue.

When plants are harmed by any kind of stress, infection by pathogens, or wounding, they start forming unorganized, undifferentiated cells that form so-called “callus”. The term “callus” derives from “callum”, a Latin word meaning hard. Originally “callus” was associated with severe cell growth caused by injuring of plant tissue. In the present time, this term is more widely applicable and defines a mass of disorganized cells. The description “callus” not only encompasses undifferentiated, disorganized cells, but also those with diverse extents of differentiation. With respect to their macroscopic properties and variability, callus cells can be categorized in several types. Undifferentiated cells, which do not form any visible organ, are named compact or crumply callus (Ikeuchi et al., 2013). Cells showing extents of organ regeneration are called shooty, rooty or embryonic callus, in accordance to which organ is formed (Frank et al., 2000).

To initiate plant cell cultures, several steps under *in vitro* culture conditions are required.

First, surface-sterilized plant parts are cut into pieces and these primary explants are placed on media containing auxins and/or cytokinins, the two types of plant growth hormones most used for callus formation (Ikeuchi et al., 2013; Mustafa et al., 2011). To induce the formation of undifferentiated cells, a 1:1 ratio of auxin to cytokinin must be used. A high ratio of cytokinin to auxin, or otherwise, results in organ formation (Skoog and Miller, 1957).

Cytokinins, derivates of N6-substituted adenine, play an important role in the modulation of cell growth. Their functions are to control cell division and the cell cycle. Various essential mechanisms of the plant growth and development are initiated and affected by cytokinins. For example, shoot initiation and xylem formation, responses to both biotic and abiotic factors, mineral absorption, leaf senescence, apical dominance and many other physiological processes are influenced by these phytohormones (Rashotte et al., 2003; Sakakibara et al., 2006; Schaller et al., 2014).

Auxins are phytohormones with a scaffold of an aromatic ring and a carboxylic group (Ljung, 2013). As an organic acid auxin plays an important role in the development and growth of

plants and controls the interaction between the plant and its environment (Friml, 2003). Moreover, cell expansion, cell division and also cell extension are influenced by auxin (Ljung, 2013), which has great impact on the plant shape as well (Jaillais and Chory, 2010). The most common auxin is indole-3-acetic acid (Vogel, 2006).

As mentioned earlier, callus formation can be induced on surface-sterilized explants on nutrient medium (Mustafa et al., 2011). An explant can also be obtained from aseptically germinated seeds. Other plant parts like stalks, leaves or roots can be equally used for explant preparation and subsequent callus induction (Smith, 2012). Depending on the type of explant, its developmental level and age, callus growth and development can vary (Dhar and Joshi, 2005; Holme and Petersen, 1996).

For the production of plant-derived SMs, different plant-based *in vitro* cultures such as cell suspension cultures, callus cultures and organ cultures are used.

Scientists have most investigated the production of SM by means of cell suspension cultures as it is principally feasible to culture them in bioreactors at industrial levels. Typically bioreactors can be stirred tank reactors, bubble column or airlift reactors (Schlatmann et al., 1996).

Starting cell suspension cultures requires the transfer of callus cells into liquid medium, which is agitated on a rotary shaker or stirred in fermentors (Hellwig et al., 2004; Mustafa et al., 2011). A few days after initiation, aggregates formed by cell suspension cultures are visible, although fine cell suspension cultures are desirable. A better reproducibility is usually achieved using cell suspension cultures comprising finely dispersed cells. Some techniques like filtration through a sieve, pipetting, or supplementing the nutrient medium with pectinase can be applied to obtain fine cell suspensions. A schematic view of establishment and maintenance of cell suspension cultures is given in Figure 3.

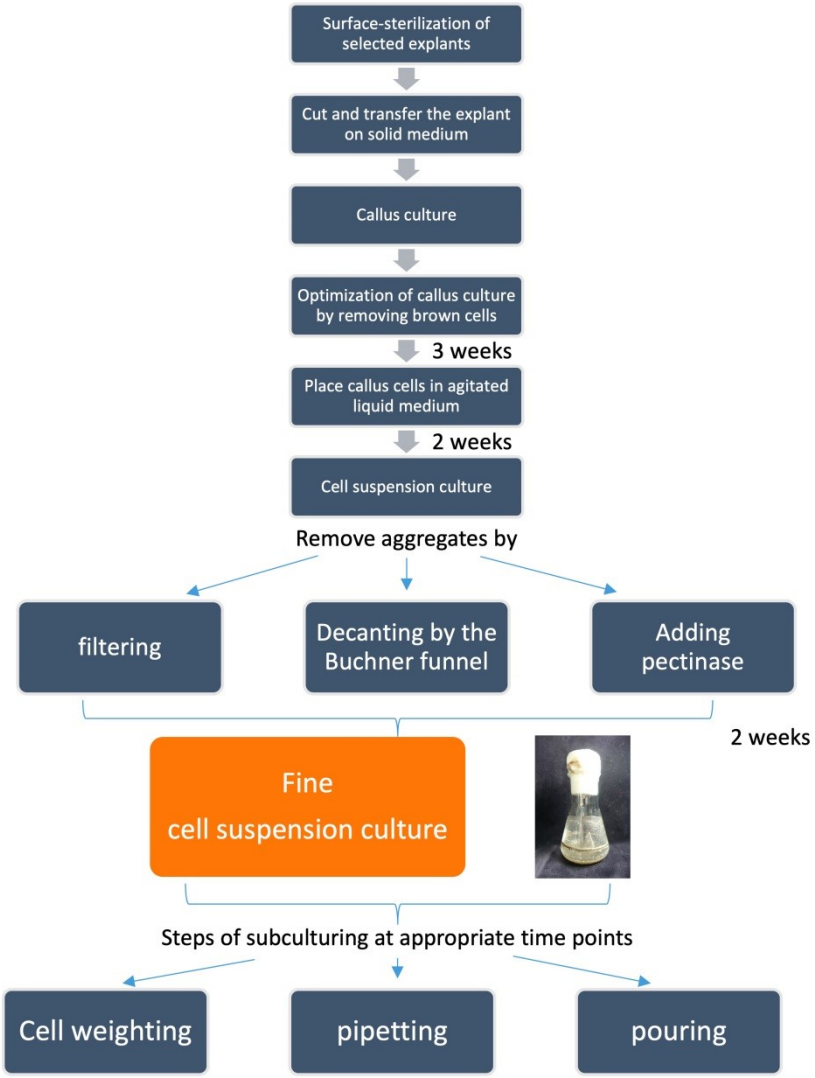

Figure 3: Procedure for the establishment of cell suspension cultures (adapted from Mustafa et al., 2011).

For the characterization of cell suspension cultures the determination of a growth curve is required. Usually, three different phases in the growth curve can be discerned, i.e. the lag phase, exponential growth phase and the stationary phase (Figure 4). However, it is known that kinetics of different cell lines vary from each other (Schripsema et al., 1990).

In the first two phases, cells start to grow and to divide by consuming nutrients necessary for the primary metabolism. A flattening of the growth curve is associated with the depletion of nutrients.

For research on cell suspension cultures and their production of SM, it is essential to work with cells at a definite stage of their growth (Smith, 2012).

According to Bourgaud et al. (2001), the highest amount of SM is produced in the stationary, or plateau phase.

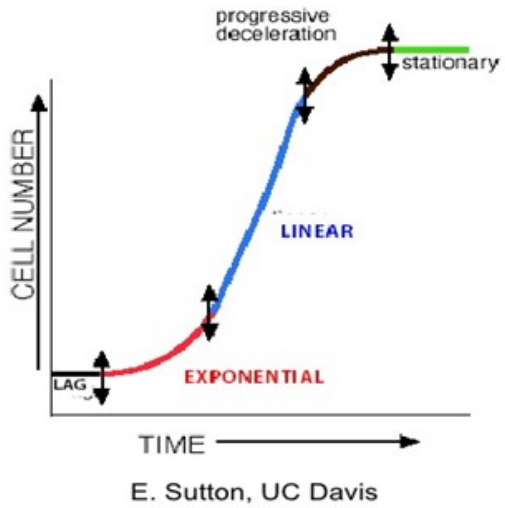

Figure 4: A prototypical growth curve of plant cell cultures (https://www.slideshare.net/SuryaSingh2/in-vitro-plant-development, retrieved on September 10, 2019).

The strategy of elicitation of plant cell cultures turned out to be very successful in some cases as it resulted in an enhanced production of SM in plant *in vitro* cultures (Bourgaud et al., 2001; DiCosmo & Misawa, 1995; Hussain et al., 2012; Jeong and Park, 2006; Karuppusamy, 2009). Another advantage of treating plant cell cultures with elicitors is the reduction of fermentation time (Jung et al., 2003).

Elicitors are compounds prompting the synthesis of secondary metabolites when supplementing them to a living cell system (Namdeo, 2007; Radman et al., 2003). Beyond the triggering of SM production, elicitors may interfere with essential metabolic pathways of the plant, thereby inducing stress. Thus, elicitation is a procedure of purposeful plant stimulation upon which the production of a compound, which adapts the plant to stressful situations, is triggered (Naik and Al-Khayri, 2016).

Figure 5 shows an overview of elicitors, which can be categorized into biotic and abiotic factors (Hussain et al., 2012). Biotic elicitors derive from organic sources like plant cell walls or microorganisms and might be polysaccharides like e.g. pectine, cellulose or chitin.

Moreover, several extracts of proteins, inactivated fungi and various yeast digests are known as biotic elicitors. Abiotic factors are ranged in physical and chemical factors.
Physical factors include e.g. UV light, pH or osmotic stress. Chemical factors comprise ozone or heavy metals. SMs often used as elicitors are methyl jasmonate and salicylic acid. (Bourgaud et al., 2001; Naik and Al-Khayri, 2016; Zhao et al., 2010).

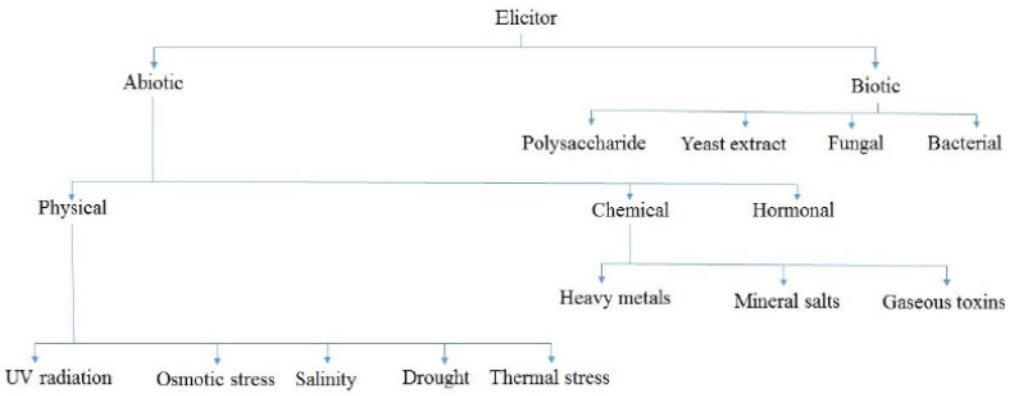

Figure 5: Classification of elicitors as biotic and abiotic factors (Naik and Al-Khayri, 2016).

2.3. Endophytes

The word “endophyte” derives from two Greek words “endon” and “phyton”, which mean “inside the plant”. Some time ago, the term was used only for fungi living within plants. This point of view has changed, as investigations showed that endophytes can be bacteria, algae and protozoans besides fungi as well (Chanway, 1996; Hardoim et al., 2015). As will be outlined below, endophytes are potential sources for new drugs (Strobel, 2003).

For a long time, vascular plants were regarded as entities living independently and autonomously (Barrow et al., 2008). Nowadays, it is known that plants do not exist on their own due to the fact that they are connected with microorganisms living nearby. Particularly, plants are associated with microbes living within them (Hardoim et al., 2015). These “domestic” organisms seem to have a symbiotic relationship with their host plants. They can be isolated from all plant tissues as they are located there during the whole plant’s life cycle or parts of it. Additionally, they are not harmful to plants or cause diseases (Sziderics et al., 2007). Investigations were done to study the phenomenon that endophytic infections linger asymptomatically. Scientists ascertained that pathogens usually break through defensive

mechanisms of the plant in such intensity that disease occurs. Compared to pathogens, endophytes overcome these mechanisms of defense to a dimension that they can contaminate and settle inside the plant.

The effects of endophytes on their host plants and how they are associated to other endophytes living in the same plant are not yet fully studied (Strobel, 2018).

Evidently, endophytes play an important role in the defense system of their host plants. They may stimulate defensive reactions of plants against pathogens, which may result in antibiosis of endophytes towards other potentially pathogenic microbes, or may alternatively trigger plant metabolites in their hosts that can augment the immune response in the plant systemically (induced systemic resistance). Antibiosis is a defense mechanism, where bacterial endophytes produce antibiotics in order to prevent the growth of invading pathogens (Kloepper and Ryu, 2006). Both ways of defensive answers are indirect. They induce an initial alert in the plant, a process called “priming”. The primed plant saves the whole information of the former excitation and reacts faster when getting in contact with the next stimulus (Martinez-Medina et al., 2016; Pieterse et al., 2014). According to Alvin et al. (2014), Strohl (2000) and Kloepper and Ryu (2006) endophytes are able to initiate the release of secondary metabolites, which stimulate or prime the defensive mechanisms of plants.

According to Strobel and Daisy (2003), endophytes generate compounds having out antibiotic, antiviral, antitumor, antioxidant, antidiabetic and immunosuppressant activities. Therefore, there is increasing interest in deploying these compounds as therapeutics against human diseases.

Another interesting fact is that microbes such as soil organisms can cope with the host’s defense system in order to colonize the plant. This can be seen in the case of leguminous vegetables and rhizobia, beneficial soil-borne microbes, which inhabit plant roots in a process called “nodulation” (Romera et al., 2019). Thereby, the two symbionts are able to interchange important nutrients. Microbes obtain their energy from plants’ photosynthetic products and fix atmospheric nitrogen into ammonium, which is useful for plants (Guinel, 2015; Hurek and Reinhold-Hurek, 2003).

Furthermore, endophytes are known for promotional effects on the plant's growth as they have inducing effects on the production of plant growth hormones (Tudzynski, 1997).

Moreover, plants are protected against soil contaminants through the natural resistance mechanisms of their endophytes. When a toxic substance enters the plant, a plant response follows, which activates defensive mechanisms of the endophytes. The presence of certain contaminants like e.g. hydrocarbons or nitroaromatics seems to lead to an enrichment in the plant of those endophyte genotypes which can degrade the respective contaminant (Siciliano et al., 2001). In recent decades there is an increasing interest in the use of plants for remediation of polluted soil, as expenses for the purification of fouled locations with conventional technologies are tremendously high (Berg et al., 2005; Kuiper et al., 2004).
Endophytes can improve the "fitness" of plants which thus become tolerant towards biotic and abiotic stress factors (Sziderics et al., 2007). Abiotic stress like e.g. drought, heat, cold or oxidative stress is the main reason for low crop yields across the globe. According to Singh et al. (2011), adaptation to abiotic stress and therefore improvement of food crop production could be promoted through the development of biotechnological applications based on endophytes.

Endophytes are able to produce metabolites such as antibiotics or enzymes with hydrolytic effects, in order to protect the plant against stress factors. A proved fact is that microbes can also generate similar compounds as their host plants. Various mechanisms have been assumed to lead to a concurrent production of such products. For example, horizontal gene transfer between the plant and the microbes could happen, which would lead to the production of the same compounds (Alvin et al., 2014).

Nevertheless, endophytes not only have a relationship to their host plant, but also among each other, the so called microbial interspecies crosstalk. Many studies claim that this kind of crosstalk contributes to the start of metabolite production in plants. These metabolites may be small molecules which act as elicitors and which lead to the start of biosynthetic processes (Kusari et al., 2012). Angell et al. (2006) proved the symbiotic interaction between endophytes and their impact on the production of metabolites. They showed that a special pigment was produced by a mixed bacterial culture, but not by a single isolate.

Another study shows the complexity of the microbial interaction and cross-talk. The compound rhizoxin, which is responsible causes rice seedling spoiling, is produced by a bacterial endophyte of the genus *Burkholderia* and not by the *Rhizopus microspores*, a fungal agent previously believed to be responsible for the phenomenon.

Understanding the biosynthesis of SM and the interaction between microbial communities is of great relevance when using endophytes for the production of desired metabolites at industrial levels. Therefore, research has to be done in uncovering the interplay between endophytes and its host plant (Partida-Martinez and Hertweck, 2005).

The most famous example for the production of the same SM by endophytes and their host plants is paclitaxel, also known as taxol, a compound with anticancer activity. The endophyte, which also generates taxol when isolated from the inner bark of the tree *Taxus brevifolia* (Pacific yew) and cultured on appropriate medium, is a fungus, called *Taxomyces andreanae*. The use of endophytes for the manufacturing of paclitaxel has been regarded as an alternative source for this valuable drug (Stierle et al., 1993).

Another representative for microbes producing the same metabolite as their host plants is the endophytic fungus *Phialocephala fortinii* recovered from the plant *Podophyllum peltatum*. The metabolite podophyllotoxin, which is generated by both the plant and the endophyte, serves as precursor for the three important anticancer drugs etoposide, teniposide, and etoposide phosphate (Eyberger et al., 2006).

An endophytic fungus isolated from the bark of the medicinal tree *Camptotheca acuminata* produces the anti-neoplastic drug camptothecin (Kusari et al., 2009). Other camptothecin producing fungi have been isolated from the Indian tree *Apodytes dimidiate* and were identified as *Fusarium solani* strains (Shweta et al., 2010). Originally, one of the most important sources of camptothecin had been *Nothapodytes nimmoniana*, an Indian tree also known as “stinking tree” (Shaanker et al., 2008). After serious over-harvesting this tree was declared as critically endangered and is therefore not available any more as source for the desired anticancer agent. Consequently, there is increasing interest in the possible suitability of endophytes for the production of camptothecin (Shweta et al., 2010).

A further example for the production of medicinally used compounds by microbes is an endophyte isolated from *Hypericum perforatum*. The fungus showed production of hypericin, an antidepressant drug, and emodin when isolated and cultured under appropriate conditions (Kusari et al., 2008).

Plants typically do not only host one type of endophytes, but various populations. Depending on geographical locations and the time of the year, the profile of endophytes can vary (Göre and Bucak, 2007). Then again, owing to the variation of populations' pattern, plants can have diverse biological activities. Kaur et al. (2016) have shown the existence of endophytes in the herb *Echinacea purpurea*, commonly applied as anti-inflammatory agent. Immunmodulatory effects of *in vitro* cultures of *E. purpurea* may be altered by these endophytes.

The formation of secondary metabolites in plant-based *in vitro* cultures can be enhanced by the use of biotic and abiotic elicitors. Selected examples for the usage of elicitors to induce the production of SM are named below:

Abiotic elicitors such as silver nitrate or sucrose are often used in order to enhance the production of desired compounds in plant cell cultures. A study by Wawrosch et al. (2014) showed that the formation of lignans in *in vitro* cultures of *Leontopodium nivale* ssp. *alpinum* increased by the use of silver nitrate as abiotic elicitor. However, the cell biomass was reduced by applying $AgNO_3$.

Similarly, hairy root cultures of *Silybum marianum* L. were treated with Ag^+ preparations of different concentrations. It was observed that the production of silymarin, a secondary metabolite used against liver diseases, was enhanced by the alteration of enzymatic activities through Ag^+ (Khalili et al., 2010). In contrast, Yan et al. (2006) found that Ag^+ decreased the production of the SM rosmarinic acid in hairy root cultures of *Salvia miltiorrhiza*. Yeast extracts (YE) as biotic elicitors were also investigated, which led to an increase in the production of rosmarinic acid in organ cultures of *S. miltiorrhiza* independently of the concentration of YE preparations.

An enhanced production of SM such as kaempferol in plant tissue cultures of *Dregea volubilis* by using YE as biotic elicitor was shown in the recent study by Yogananth et al. (2019).

Another example of biotic elicitor is methyljasmonate (MJ). Upon elicitation with MJ, the lignan production in plant tissue cultures of *Linum album* increased (Fuss, 2003).

Several studies report the enhancement of productivity of desired products in plant cell cultures especially by microbes. Some instances are described below:

Enhanced SM production in plant-based *in vitro* cultures through elicitation with endophytes was demonstrated in a study by Jung et al. (2003). The authors demonstrated that elicitors of biotic origin stimulated the generation of tropane alkaloids in plant organ cultures of *Scopolia parviflora*. Live and autoclaved bacteria were used for the treatment of hairy root cultures, while the former led to an increased production of scopolamine, the autoclaved bacteria had no impact on the alkaloid pattern.

Another example for the use of endophytes as elicitors is the work of Chen et al. (2016). Meristem cultures of the traditional Chinese plant *Atractylodes lancea*, which is used against e.g. inflammatory disorders and influenza, were treated with the endophytic fungus *Gilmaniella sp.* AL12, isolated from the stem of *A. lancea*, in order to accumulate more volatile oils, the principal biological active constituent in the plant. The results of this study were satisfactory, as an increase in the accumulation of volatile oils could be achieved.

In the study by Pawar et al. (2011), callus cultures initiated from leaf and stem explants of *Calophyllum inophyllum* and were treated with two endophytic fungi, namely *Nigrospora sphaerica* and *Phoma* sp., which were isolated from the leaves of the plant. Samples elicited with endophytes showed a higher production of inophyllum, an anti-HIV agent, compared to the control. Elicitation of callus cultures of the plant *Artemisia annua* with an endophytic *Aspergillus sp*., which was isolated from the stem of the plant, led to an increase in artemisinin formation (Yuliani et al., 2018). Enhanced production of rutin in hairy root cultures of *Fagopyrum tataricum* was achieved through elicitation with mycelia-derived polysaccharides of the endophyte *Fusarium oxysporum* (Zhao et al., 2014). Rhizomes of *Dioscorea zingiberensis* host the endophytic fungus *Fusarium oxysporium* Dzf17. Greater accumulation of the compound zingiberin in cell suspension cultures of *D. zingiberensis* was reached by adding polysaccharides of *Fusarium oxysporium* as elicitors at late log-phase (Li et al., 2011).

In an interesting approach Ding et al. (2018) worked with co-cultures of plant tissue cultures of *Rumex gmelini* Turcz (RGT) and three of its endophytes. These strains of *Aspergillus* sp., *Fusarium* sp., and *Ramularia* sp. were able to produce the same metabolites as the host plant, however the production decreased considerably after prolonged cultivation. Enhanced production of SM by RGT seedlings in co-cultures with each of the three endophytic fungi each, but especially with the *Aspergillus* strain, could be observed.

Similarly, co-culture of *Centiella asiatica* shoots with its endophytic fungus *Colletotrichum gloeosporioides* resulted in enhanced production of the plant metabolite asiaticoside (Gupta and Chaturvedi, 2019).

A study recently published was concerned with the elicitation of cell suspension cultures obtained from roots of *Withania somnifera*. Two different preparations of the plant-derived fungus *Aspergillus terreus* 2aWF were used as elicitors. Six hours after elicitation with the endophytic culture filtrate, an increased production of the plant's SM withanolide A was noticeable. Upon using the mycelial extract of *A. terreus* as elicitor, an even higher concentration of the metabolite was reached after 24 hours (Kushwaha et al., 2019). The use of endophytes as elicitors in plant tissue cultures is now regarded as a promising approach to enhance the production of desired secondary metabolites (Gupta and Chaturvedi, 2019).

3. Aim of this work

The aim of this work was to investigate whether endophytes obtained from the solanaceous plant *Atropa belladonna* may have an impact on the alkaloid production of cell suspension cultures of *A. belladonna*.

Consequently, the following experiments had to be performed:

-Establishment of cell suspension cultures

Callus cells will be added to liquid medium and grown to a homogeneous, fine cell suspension culture.

-Finding cell lines with significant alkaloid production

In order to find cell lines with a high production of atropine and scopolamine, all available cell suspension cultures will be lyophilized, extracted and then analyzed by usage of HPLC.

-Determination of growth curves

Cell lines will be characterized through their growth kinetics. Therefore, growth curves of those cell lines, which showed significant alkaloid formation, will be determined.

-Elicitation of cell suspension cultures

A cell line with significant alkaloid production and small aggregate formation on the last day of cultivation will be chosen for elicitation experiments with autoclaved endophyte preparations.

-Quantification of scopolamine and atropine by HPLC

Finally, tropane alkaloids will be extracted from lyophilized cells treated with endophyte elicitors and quantified by HPLC.

4. Materials and Methods

4.1. Plant material

For this project, callus lines were established from leaf explants of axenic *Atropa belladonna* seedlings in April 2018 by Mag. Silvia Löbsch (Department of Pharmacognosy, University of Vienna). One callus line (AB 2) has been initiated in October 2016 by Dr. Christoph Wawrosch (Dept. of Pharmacognosy, University of Vienna) from surface sterilized petiole explants of an A. belladonna plant grown in the medicinal plant garden of the Department. All callus lines were maintained on medium B (see chapter 4.3).

4.2. Microbial material

Bacterial endophytes of *A. belladonna* were isolated from three plants grown in the medicinal plant garden of the Department of Pharmacognosy, University of Vienna, by Dr. Martina Oberhofer in 2016. Subsequently, a number of isolates was identified by Salem (2018) and Oberhofer (personal communication) through 16S rDNA sequencing. The endophyte strains used for elicitation studies within the present Thesis were chosen based on results from a previous elicitation experiment on *A. belladonna* callus cultures with a large number of bacterial endophytes in an earlier Diploma Thesis. The four bacterial strains chosen for this Thesis showed the largest impact on tropane alkaloid production and are described in chapter 4.7.1.

4.3. Culture media

For the propagation and maintenance of callus and cell suspension cultures of *A. belladonna*, MS medium (Murashige and Skoog, 1962) supplemented with plant growth regulators was used as basal medium. For media preparation 100 –fold concentrated stock solutions of the macro-elements and the sodium Fe^{2+}- EDTA while the stock of the micro-elements was 1000-fold concentrated and the solution of vitamins and glycin was 200-fold concentrated. The stock solutions of the plant growth regulators such as kinetin (Sigma-Aldrich, K-0753) and 1-naphtaleneacetic acid (NAA; Sigma-Aldrich, 201-705-8) were 1 mM. All solutions were stored

in the fridge except the KNO_3 stock, which was kept at room temperature. All chemicals were of standard biochemical grade except sucrose, which was of household quality.

The two nutrient media used throughout our studies were the semi-solid medium B with 10 µM NAA and 2.5 µM kinetin, and the liquid medium H with 20 µM NAA and 0.5 µM kinetin (Salem, 2018). Medium B was fortified with 3 g/l Gelrite™ (Roth, 71010-52-1).

For media preparation, appropriate aliquots of the stock solutions were mixed with the necessary amount of distilled water. Sucrose and myo-inositol as well as gelrite for semi-solid medium were added and the pH value was adjusted to 5.7±0.1 with KOH.

Small HIPP™-jars (65mm in height, 55mm in diameter) were filled up with 40 ml semi-solid medium B and closed with Magenta™ B-caps. For liquid medium H, 50 ml were dispensed into 250 ml Erlenmeyer flasks and closed with cellulose plugs. Media were then autoclaved at 121° C for 20 minutes and stored at 5° C until use.

4.4. Culture conditions

The callus cell lines on medium B were regularly sub-cultured every four weeks and were kept at 25±1° C in the dark. Cell suspension cultures were placed on a rotary shaker at 120 rpm at 25±1° C in the dark. They were sub-cultured every two to three weeks by filtering off the cells with the help of a Buchner funnel and transferring an appropriate amount of cells into 50 ml of fresh medium.

4.5. Establishment of cell suspension cultures

To establish cell suspension cultures, callus cells cultivated on semi-solid medium B were transferred to a 250 ml Erlenmeyer flask with 50 ml of liquid medium H. The flasks were placed on a rotary shaker at 120 rpm at 25 ± 1° C in the dark.

After approximately 20 days, large aggregates were dislodged by the usage of a tea sieve and the medium with single cells and smaller aggregates was transferred to a sterile flask. About ten minutes later, the supernatant was removed with a pipette after the cells had settled on the bottom of the flask. 20 ml of this sieved suspension were then added to a 250 ml Erlenmeyer flask containing 50 ml of fresh medium H.

After four weeks, the suspension cultures had to be transferred into new, fresh medium again. To this purpose, the cell suspension cultures were filtered using a Buchner funnel and 3 g of nearly dry cell mass were transferred into 250 ml Erlenmeyer flasks containing 50 ml

of medium H. Sub-culturing and maintenance of cell suspension cultures was performed as described in chapter 4.4.

4.6. Determination of a growth curve of cell suspension cultures

18-day-old cell lines were first filtered using a Buchner funnel. Afterwards, three grams of cell mass were added to each flask containing 50 ml of medium H. Thirty flasks per cell line were inoculated this way and were kept on a rotary shaker as described.

Every second day the cell biomass in three flasks of each cell line was determined. This was accomplished by transferring the content of one flask to a tared centrifuge tube and weighing of the pellet after centrifugation for 20 minutes at 4000 rpm. Subsequently, the cells were frozen at -20 °C, lyophilized, and weighed again to obtain the dry weight.

This procedure was repeated ten times so that a growth curve over 19 days could be plotted.

4.7. Elicitation

4.7.1. Endophyte strains

For elicitation studies, four endophytes isolated from *Atropa belladonna* L. by Dr. Martina Oberhofer (see chapter 4.2.) were chosen. They differed in their growth rates and thus in the time span reaching stationary phase after inoculation in sterile tryptic soy broth (TSB) (Table 1). Isolates AB 35, AB 80, AB 79 and AB 7 were chosen as elicitors, two of which are Gram-positive bacteria belonging to Actinobacteria. The other two are Gram-negative bacteria, whereby *Bosea* belongs to alpha proteobacteria and can fix nitrogen from the air and the other belongs to Gammaproteobacteria. Preparations of endophytic elicitors were used for the treatment of cell suspension cultures of *A. belladonna*.

Table 1: Bacterial strains and their properties applied as elicitors on cell suspension cultures of *Atropa belladonna* (information supplied by Mag. Dr. Martina Oberhofer).

Number of isolate	Blast ID	Approximate time to reach the stationary growth phase (h)
AB 80	*Bosea* sp.	96
AB 35	*Pseudomonas* sp.	30
AB 79	*Microbacterium arthrospheriae*	76
AB 7	*Promicromonospora* sp.	30

4.7.2. Determination of a growth curve of bacterial cultures

Approximately one week before measuring the growth curves of bacteria, a fractionated streak out of every endophytic strain was done on TSA medium. After the colonies reached an adequate size, one colony per strain was picked to ensure a pure culture going back to a single cell. With respective colonies 2 ml of liquid TSB medium were inoculated and incubated at 28 °C overnight in the shaking incubator at 200 rpm, this was used as seed culture. Subsequently, Erlenmeyer flasks were filled with 60 ml TSB medium and were inoculated with 10 µl of each of the seed cultures. As a blank for the further growth curve measurement, another flask was incubated with TSB and served as negative control. The flasks were then kept in the shaking incubator at 200 rpm and 28 °C.

24 hours after inoculation, the bacterial strains' optical density was measured three times a day with intervals of 2 hours with a photometer at 600 nm. The growth curve measurements corresponding to each strain ended after reaching the stationary phase (instructions were given by Mag. Dr. Martina Oberhofer).

4.8. High Performance Liquid Chromatography (HPLC)

For the quantification and identification of tropane alkaloids in cell suspension cultures of *A. belladonna* after 48 hours of elicitation, High Performance Liquid Chromatography was utilized as analytical method.

4.8.1 Sample preparation

For HPLC analysis of tropane alkaloids, cells of *Atropa belladonna* were extracted as described by Salem (2018).

The lyophilized cells obtained from one culture flask (at least 100 mg and up to 800 mg) were ground in a mortar and a weighed amount of the fine powder was wetted with methanol. After addition of 10 ml 1M HCL the mixture was sonicated for 20 minutes at 21° C. After centrifugation the extract solution was filtered into a separation funnel, the tube and the filter were washed with 5 ml of distilled water. The filtrate was alkalized with concentrated ammonia to pH 10 and was then extracted three times with 10 ml chloroform each. The organic phase was filtered through cotton wool to remove residual aqueous phase and was evaporated to dryness under reduced pressure at 40 °C. The residue was then dissolved in 1.00 ml of methanol and centrifuged at 13,000 rpm for 10 minutes prior to HPLC analysis.

4.8.2. HPLC device and conditions

The HPLC equipment (Shimadzu, Kyoto) used for the quantification of scopolamine and atropine in *in vitro* cultures of *Atropa belladonna* was adopted from Salem (2018) and consisted of a DGU-20A5R degasser, a LC-20AT pump, a SIL-20AC HT auto sampler, a photodiode array detector, a column oven from model CTO-20AC and a CBM-20A controller.
A Luna®C18 (2) column (Phenomenex Ltd., Aschaffenburg, Germany) with a length of 250 mm, a particle size of 5 µm and a diameter of 4.6 mm was employed.

The HPLC method for identifying and quantifying the tropane alkaloids in the samples was developed by Salem (2018). Table 2 lists the solvent concentrations for the gradient elution

program. An aqueous solution of 0.01% formic acid (reagent grade, Sigma Aldrich) was used as solvent A while acetonitrile (HiPerSolv CHROMANORM® Super gradient for HPLC, VWR chemicals) was applied as Solvent B. The injection volume of the extract solution was 100 µl and every sample was analyzed three times.

Table 2: HPLC gradient for tropane alkaloid analysis (Salem, 2018).

Time (min)	Solvent A (%V/V)	Solvent B (%V/V)
0.00	95	5
17.00	35	65
18.00	5	95
23.00	5	95
24.00	95	5
34.00	95	5

4.8.3. Alkaloid identification and quantification

For the identification and quantification of atropine and scopolamine, the chromatographic data were processed with the software Labsolution, Version 5.82 (Shimadzu Europa GmbH). Peaks were integrated valley-to-valley by using the software tool "manual integration".

For the quantification via external standard a solution of 2.5 µg/ml each of atropine (Sigma Aldrich, Y0000878) and scopolamine hydrobromide (Sigma Aldrich, H1500000) in methanol was prepared. The injection volume of this standard solution was 10 µl and the average peak areas of three analyses were used to calculate the alkaloid contents of the samples. As scopolamine hydrobromide (MW 384.26) was used as reference, the calculated content after HPLC analysis was multiplied with the factor 0.789 to obtain the actual value for the alkaloid base (MW 303.35).

For the generation of calibration curves of the alkaloids, methanolic solutions of atropine (2.558, 5.116, 51.16, 255.8, 511.6 µg/ml) and scopolamine hydrobromide (2.525, 5.050, 50.50, 252.5, 505.0 µg/ml) were used. Considering the low alkaloid content in *A. belladonna* cell cultures, as compared with the study of Salem (2018) the 2.5 µg/ml dilution was included. Each solution was analyzed six times, with an injection volume of 10 µl. The

compilation of the calibration curves and the calculation of the correlation coefficients were accomplished with Microsoft Excel 2010.

4.8.4. Statistical analysis

All HPLC results were statistically evaluated using the software Statistica version 6 (StatSoft Europe GmbH). Analysis of variance followed by Duncan's Multiple Range Test ($p \leq 0.05$) was applied to determine significant difference between treatment means. The HPLC calibration curves for atropine and scopolamine were created with Excel 2010 (Microsoft Corporation).

5. Results

5.1. Establishment of cell suspension cultures

20 callus cell lines maintained on semi-solid medium B (10 µm NAA and 2.5 µM kinetin) were provided by Mag. Silvia Löbsch and Dr. Christoph Wawrosch (Department of Pharmacognosy, University of Vienna) as starting material. To obtain cell suspension cultures with a fast increase of biomass, three to four grams of callus were added to a 250 ml Erlenmeyer flask containing 50 ml of medium H (20 µM NAA and 5 µM kinetin). For propagation and maintenance, the content of the flasks had to be sub-cultured every three to four weeks. Some cell lines showed aggregate formation, thus a tea sieve with a mesh size of 1.5 mm was used to remove large aggregates. 20 ml of the resulting fine suspension were then transferred to 50 ml of fresh medium H. An inoculum of 10 ml was also tested as starting volume but a suboptimally growing cell suspension culture with brown color was obtained.

After 13 days on average, fresh liquid medium (20 ml) was added to fine cell suspension cultures in order to keep the cells in an actively growing state.

Subsequently, for sub-cultivation the cell suspension cultures were filtered with the help of a Buchner funnel. Three grams of cells were then routinely used to inoculate 50 ml of nutrient medium.

After several steps of passaging, six suspension culture lines established from the initial 20 callus lines showed very fast growth with minor aggregate formation and ivory-colored

appearance. One cell line grew very fast, was ivory colored but formed small aggregates. Four lines grew slower and displayed hard cell clumps and a brownish color. Two cell lines grew slower without forming any aggregates. One of them was dark-colored and one was ivory-colored. Three cell lines grew very slowly, were brown- to dark brown-colored and produced big aggregates. The filtering step of these cell lines was very difficult as the nutrient medium developed a significant viscosity and clogged the filter paper.
Four cell lines were obviously contaminated after some time, since their growth ceased and the cultures were orange-colored. A viability test with fluorescein diacetate (Widholm, 1972) revealed that all cells were dead, and thus these lines were discarded.

5.2. HPLC calibration curves

The suitability of the HPLC method for the quantification of tropane alkaloids in cell suspension cultures was verified through the generation of calibration curves for atropine and scopolamine. Serial dilutions of methanolic stock solutions of the two compounds were prepared and analyzed 6 times with HPLC. Subsequently, mean values and standard errors were calculated and the curves were plotted (Figures 6 and 7).
The calibration curves were determined by area under the peak of each standard against the corresponding concentration. On the basis of the measured values k, d and the correlation coefficient were calculated by using MS Excel. The higher the correlation coefficient, the more linearity is given.

A linear equation of y = 19780x – 52793 and a correlation coefficient of 0, 9987 were specified for the calibration curve of atropine.
For the calibration curve of scopolamine, a linear equation of y = 12187x + 26250 and a coefficient of correlation of 0, 9992 were defined.

These results confirm the findings of Salem (2018), and by including a 2.5 µg/ml dilution we could ensure the suitability of the HPLC-based quantification method at low alkaloid concentrations which were to be anticipated in *A. belladonna* cell cultures.

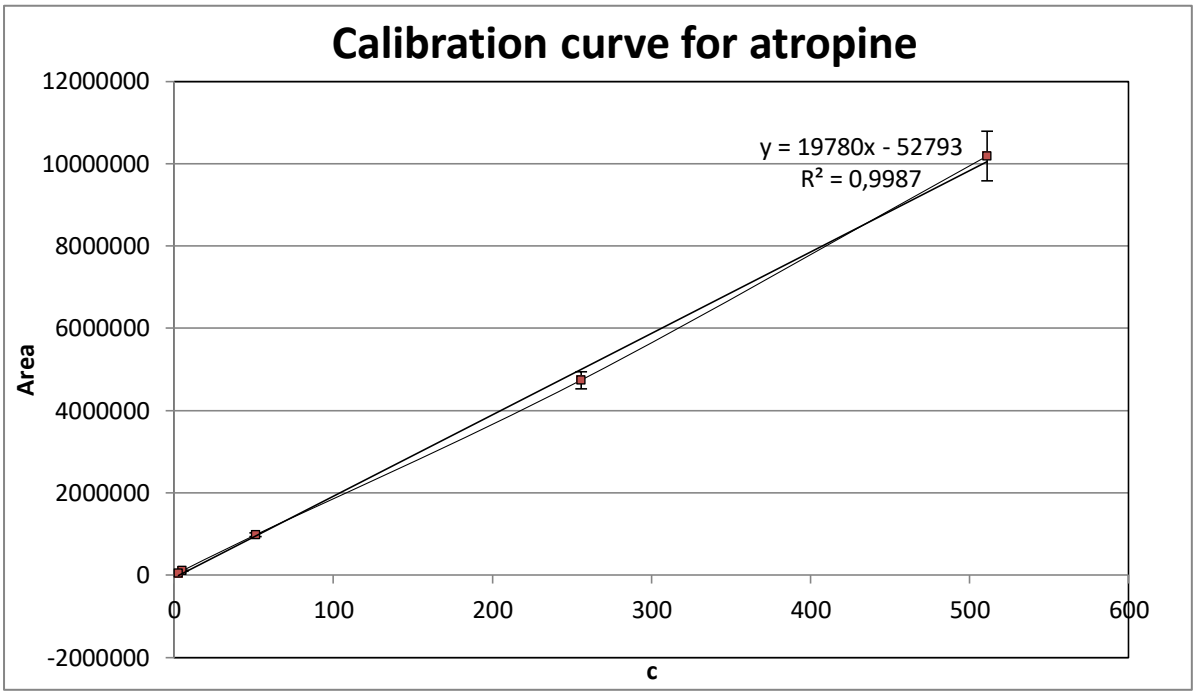

Figure 6: Calibration curve of atropine with solutions of 511.6; 255.8; 51.16; 5.116; 2.558 µg/ml, means with standard error.

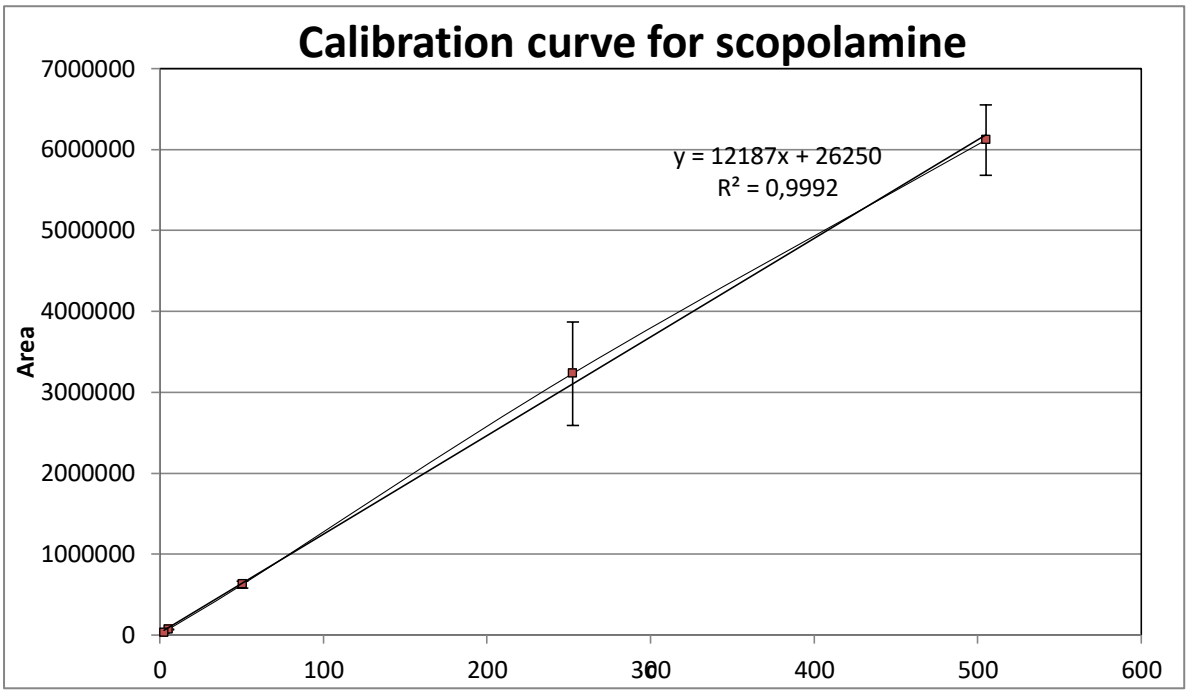

Figure 7: Calibration curve of scopolamine with solutions of 505.0; 252.5; 50.50; 5.050; 2.525 µg/ml, means with standard error.

5.3. Tropane alkaloid formation in cell suspension cultures

For the determination of the alkaloid content in the cell suspension cultures, the cells of one flask of each of the lines were separated from the medium through centrifugation and frozen at -20° C. After lyophilization the cells were extracted as described in chapter 4.8.1. and the extracts were then analyzed by HPLC. Table 3 lists the 20 cell suspension lines with their respective contents of atropine and scopolamine, their growth performance, degree of aggregate formation and occurrence of brown discoloration. Discarded cell lines are also mentioned as they were contaminated after determining their alkaloid contents. Unfortunately, given the limit of quantification of 10.22 µg/ml for atropine and 6.76 µg/ml for scopolamine (Salem, 2018), all but 2 values (31.55 µg/g atropine in line AB 8BW and 12.58 µg/g scopolamine in line AB 9BW) were below the respective LOQ.

Based on alkaloid content, growth and aggregate formation five cell lines were selected for further experiments, these lines are presented in table 3.

Table 3: Alkaloid content and properties of cell suspension cultures of *Atropa belladonna* L. (nd = not detectable).

Cell line	Atropine (µg/g)	Scopolamine (µg/g)	Growth	Aggregate formation	Browning	Infection
AB1	3.71	4.39	+++	-	-	
AB 13BK	**2.19**	**0.43**	**++**	**-**	**-**	
AB 13 W	nd	nd	+	+	++	*
AB 14K	0.92	1.19	+	+	++	*
AB 17	8.83	2.30	+	+	++	*
AB 2	**1.90**	**1.88**	**+++**	**+**	**-**	
AB 2W	**1.14**	**0.29**	**+++**	**-**	**-**	
AB 4	1.93	5.29	++	+	++	
AB 6	4.27	0.35	+	+	++	*
AB 6W	0.36	0.39	++	+	++	
AB 7AW	nd	nd	++	++	++	
AB 7BW	nd	nd	+	++	++	
AB 7W	8.21	6.07	+	++	++	
AB 8BW	**31.55**^{LOQ}	**2.55**	**++**	**+**	**++**	
AB 9AW	nd	nd	++	-	++	
AB 9D	3.27	1.56	+++	-	-	
AB 9DW	**0.29**	**0.13**	**+++**	**-**	**-**	
AB 9W	1.74	0.13	+++	-	-	
AB 9BW	3.03	12.58 ^{LOQ}	+++	-	-	
AB 14AK	n.d.	n.d.	+	++	+	

^{LOQ} value above LOQ

5.4. Growth curves for the cell suspension cultures

In order to determine a growth curve for each of the 5 cell lines chosen (see chapter 5.3.), 30 Erlenmeyer flasks per cell line containing 50 ml of fresh medium H were inoculated with three grams of cells and incubated on the rotary shaker.

Every second day, three flasks of each cell line were harvested and the wet weight of the cells was determined over a period of 19 days. Each sample was frozen at -20 °C and subsequently lyophilized to obtain the dry weight.

In the figures 8 to 12 it is difficult to see that besides the increasing fresh weight the dry weight also increases. However, as a rule the trend of dry weight is in line with that of the fresh weight.

Figures 8 to 12 show the growth curves based on fresh weight (FW) and dry weight (DW) of the 5 cell lines.

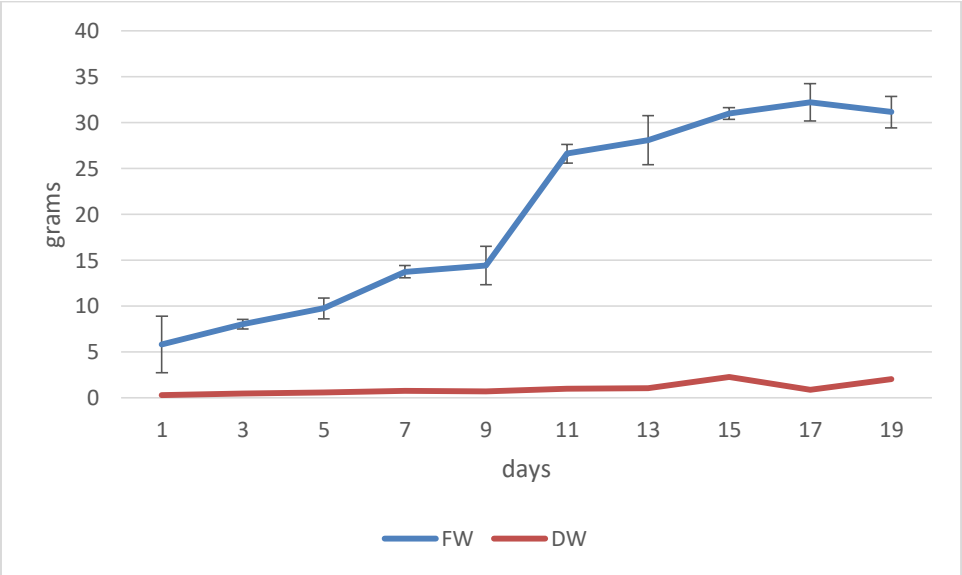

Figure 8: Growth curves of cell line AB 2 (n=3).

On day 19, cell line AB 2 formed aggregates of 1-2 mm.

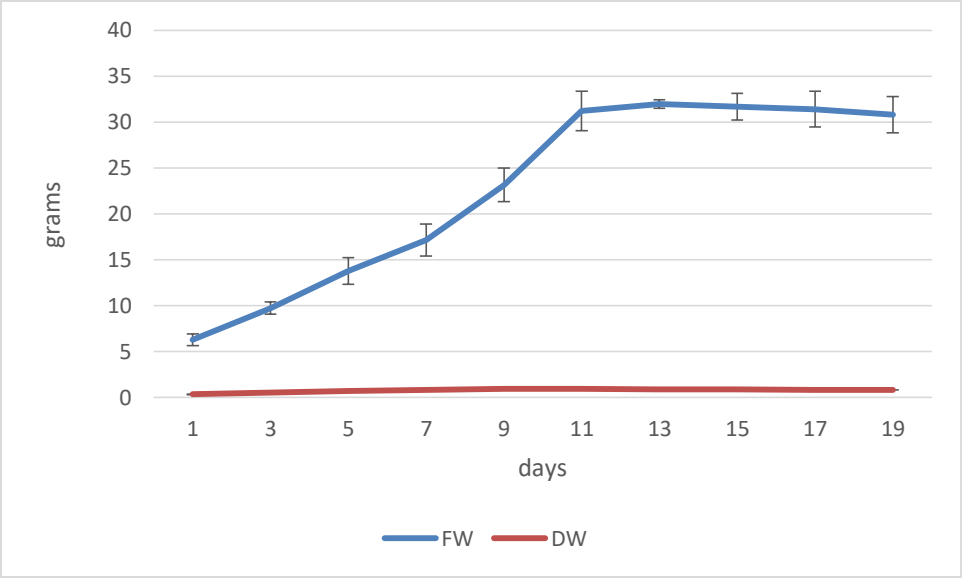

Figure 9: Growth curves of cell line AB 8BW (n=3).

On day 19, cell line AB 8BW showed aggregates of 1-3 mm.

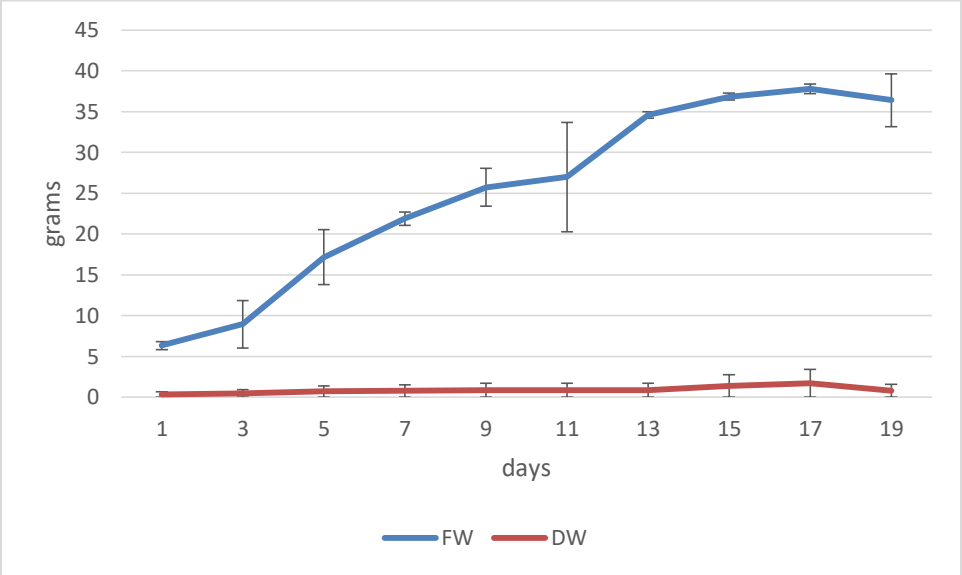

Figure 10: Growth curves of cell line AB 2W (n=3).

On day 19, cell line AB 2W showed no aggregate formation and was of very thick consistency.

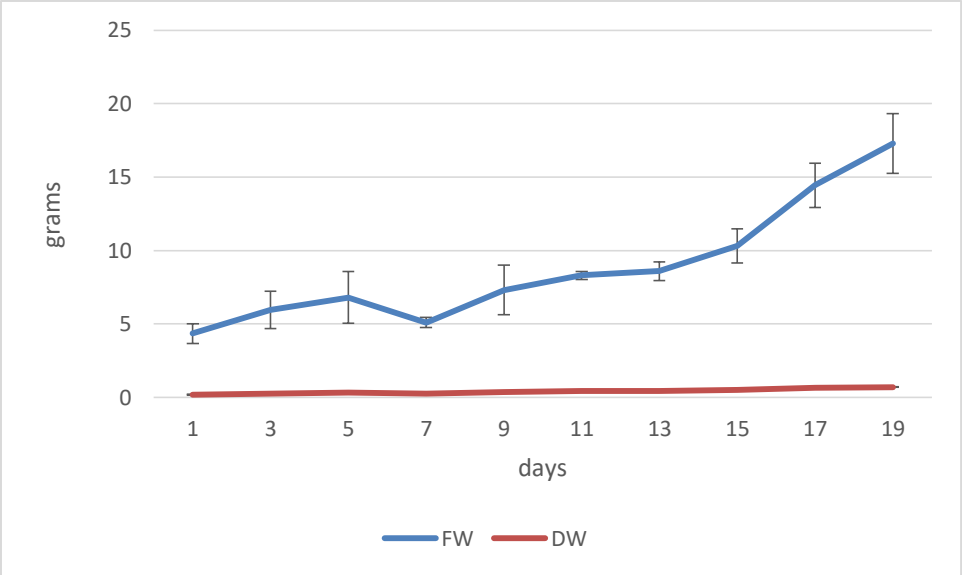

Figure 11: Growth curves of cell line AB 13 BK (n=3).

On day 19, cell line AB 13 BK was a very thin suspension without any aggregates, which got brownish-colored after several days of incubation. A (typically brown) coloration can be signify dead or harmed cells (Htwe et al., 2011; Li et al., 2016). Compared to the other cell lines AB 13 BK showed only poor growth.

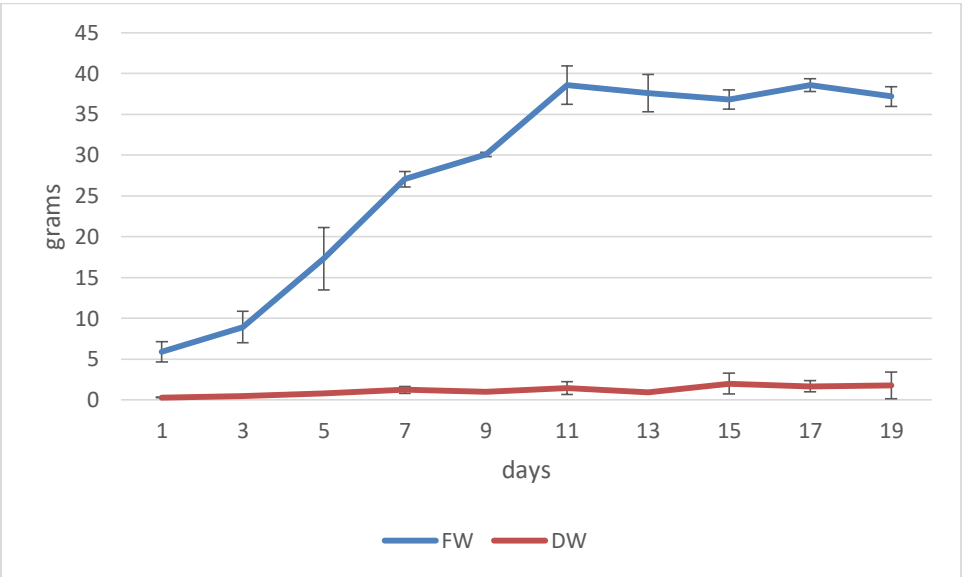

Figure 12: Growth curves of cell line AB 9DW (n=3).

On day 19, the formation of very large cell aggregates was apparent in line AB 9DW.

After considering all the facts, cell line AB 8BW was chosen for the elicitation experiments as it showed the highest alkaloid production and grew fast with high biomass accumulation and formation of only small aggregates.

5.5. Initiation and growth determination of bacterial cultures

For elicitation studies, four endophyte strains isolated from *A. belladonna* by Dr. Martina Oberhofer were chosen. The strain AB 80 belongs to the genus *Bosea*, AB 35 to *Pseudomonas*, AB 79 to *Microbacterium* and AB 7 to *Promicromonospora*. A fractionated streak out of each endophytic strain was done on TSA medium.

After one week, seed cultures were established by picking colonies from the streak out plates and inoculating them in TSB medium. Subsequently, for each strain 60 ml of TSB medium were inoculated with 10 µl of the seed culture and were kept at 28 °C in the shaking incubator at 200 rpm. Exactly one day after inoculation, the optical density of each bacterial strain was measured every two hours with the photometer at 600 nm. Initially, the optical density of strains AB 35, AB 7 and AB 80 increased fast. However, it decreased again after four to five measurements. The optical density of AB 79 showed a classical increase during all measurements without any decrease.

Figures 13 to 16 show the growth curves of the different bacterial strains.

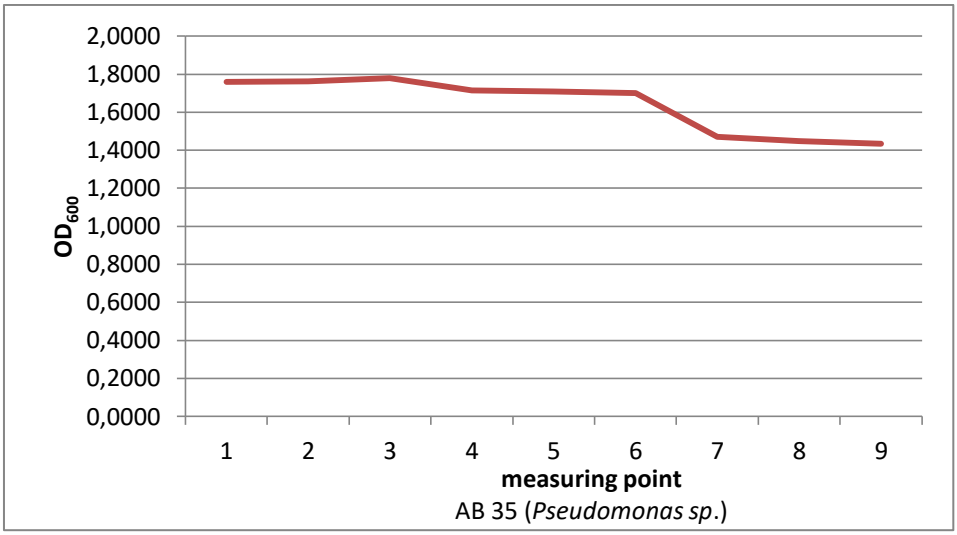

Figure 13: Growth curve of bacterial strain AB 35.

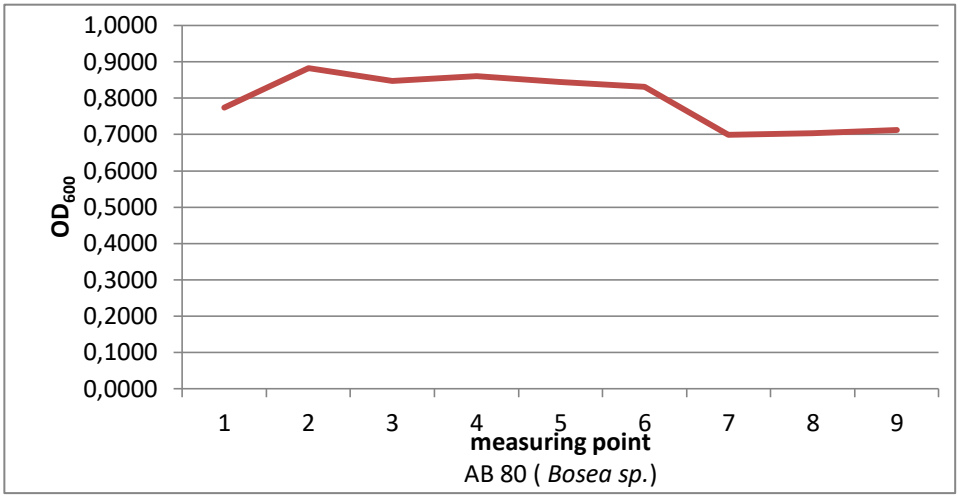

Figure 14: Growth curve of bacterial strain AB 80.

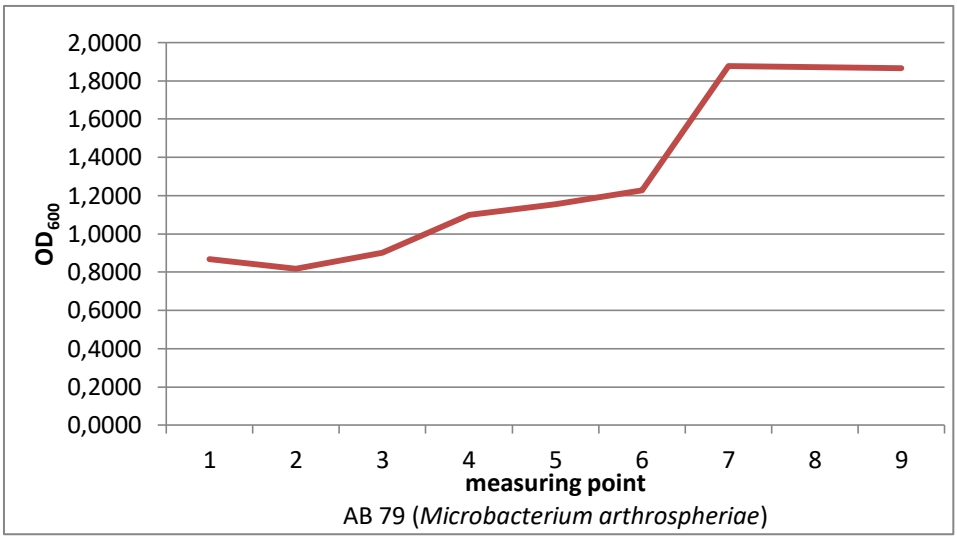

Figure 15: Growth curve of bacterial strain AB 79.

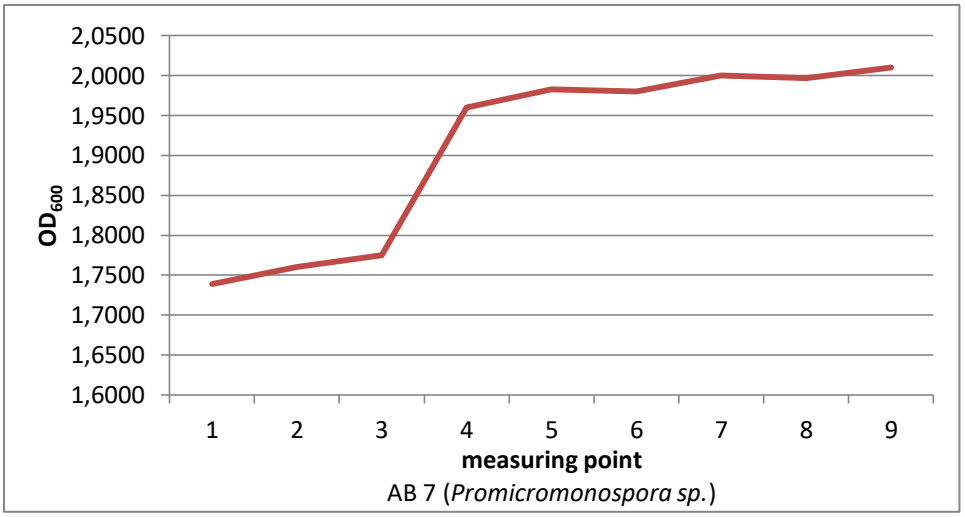

Figure 16: Growth curve of bacterial strain AB 7.

5.6. Elicitation

Figure 17 gives a general overview of the alkaloid pattern of the 7-day-old suspension cell suspension culture line AB 8BW of *A. belladonna* 48 hours after elicitation. They were treated with different preparations such as the pellet (P) and the supernatant (S) of bacterial cultures.

5.6.1. Elicitation treatments

30 ml of each bacterial culture growing in TSB medium were filled into Falcon® tubes and were centrifuged. The supernatant (elicitor S) and a suspension of 1 ml pellet in 5 ml of water (elicitor P) were stored at -20 °C until use.
For the elicitation experiment, 5 ml of autoclaved elicitor S as well as a dilution of 1 ml autoclaved elicitor P with 4 ml of water were used as follows.

Experimental design of the elicitation procedure:

- 3 Erlenmeyer flasks of 7-day old cell suspension culture with 5 ml of elicitor S of each bacterial culture.
- 3 Erlenmeyer flasks of 7-day old cell suspension culture with 5 ml of elicitor P of each bacterial culture.

- 3 Erlenmeyer flasks of 7-day old cell suspension culture with 5 ml TSB-medium as vehicle-control.
- 3 Erlenmeyer flasks of 7-day old cell suspension culture with 5 ml autoclaved water as water-control.
- 3 Erlenmeyer flasks of 7-day old cell suspension culture as untreated control.

In total 33 flasks containing treated and untreated suspension cultures were kept on the rotary shaker at 115 rpm and 25 °C for 48 hours. Subsequently the cells were harvested as described in chapter 4.6.

Compared to the untreated control and the water control a significant decrease of atropine is noticeable in cultures treated with bacterial pellets in Figure 18.
In Figure 19, cultures treated with the supernatants of different bacterial strains show significantly reduced atropine content as compared to the untreated control. However, the TSB control treatment likewise caused a significant inhibition of atropine production.

Compared to the untreated control and the water control a significant increase of scopolamine was noticed in cultures treated with the pellet of bacterial strain AB 35. Enhanced scopolamine production was also induced upon treatment with pellet of strain AB79, although the effect was not statistically significant. Cultures treated with the pellets of the other strains showed no significant difference in the scopolamine content compared to the water control and the untreated control (Figure 20).

The most interesting observation was the impact of the bacterial supernatant on the scopolamine content of the cell suspension culture. The TSB control alone led to a pronounced stimulation of scopolamine formation. AB 7 induced a further increase in the scopolamine content when compared to the TSB control, but this difference was not significant. Treatment with AB35 S resulted in the highest scopolamine concentration while AB79 S and AB80 S resulted in scopolamine amounts lower than in the TSB control, but higher than in the untreated samples (Figure 21).

The wet and the dry weights of treated cell suspension cultures were also determined. After statistical analysis, it was seen that the values obtained after elicitation were not significantly different from each other. Figures 22 and 23 show the impact of elicitation with different bacterial strains on the dry and wet weights of the 7-day-old cell suspension culture.

In summary, both treatments with the bacterial pellets and the supernatants led to a decrease in total alkaloid contents, but the ratio of atropine to scopolamine was massively shifted towards the latter compound.

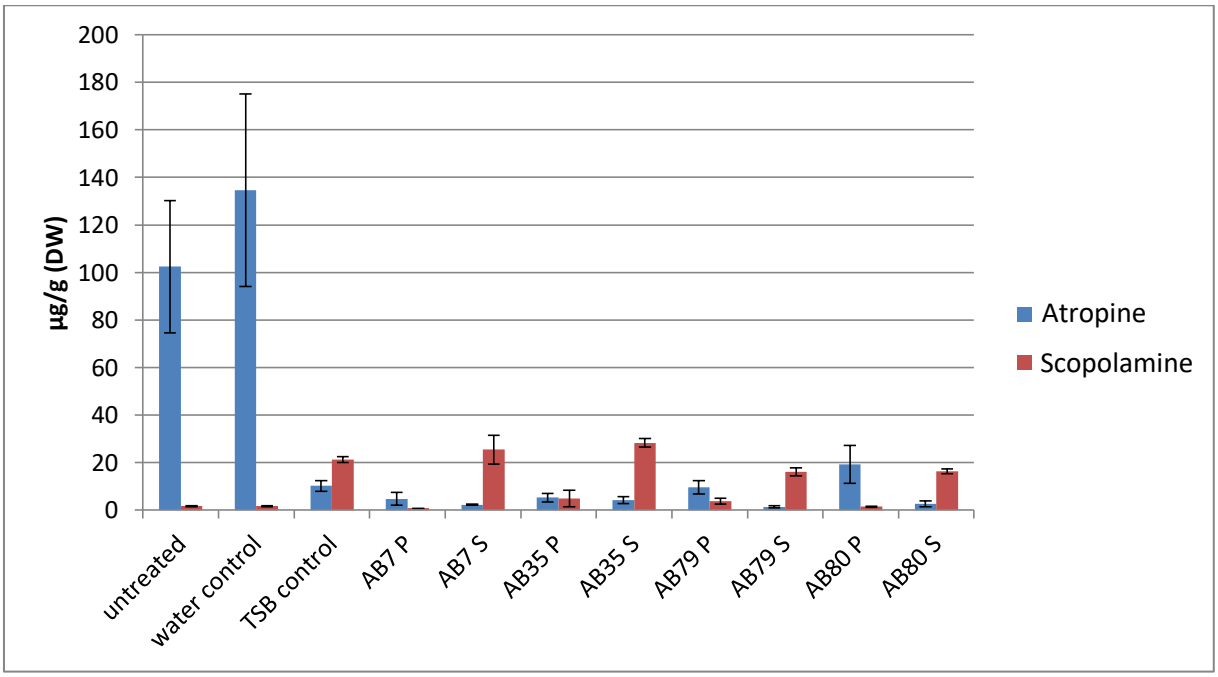

Figure 17: An overview of the alkaloid content of a 7-day-old cell suspension culture of *A.belladonna* 48 hours after elicitation with the pellet (P) and the supernatant (S) of different bacterial strains (n=3).

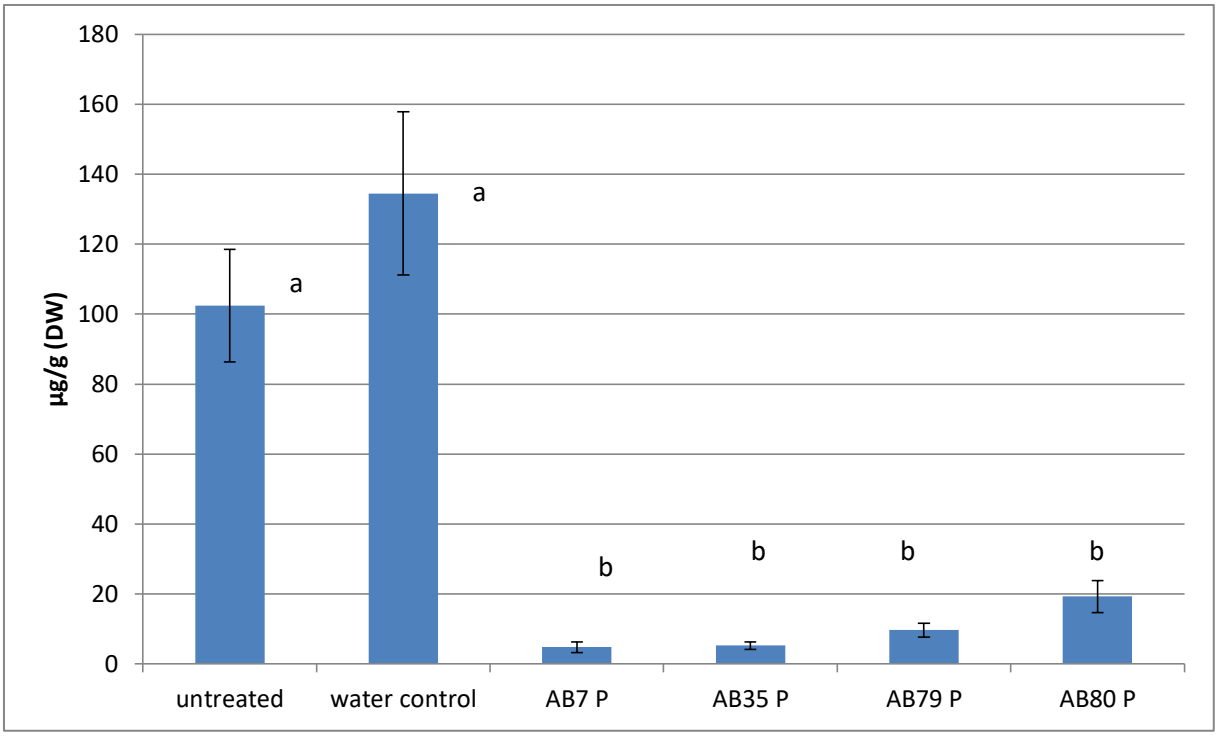

Figure 18: Atropine content of a 7-day-old cell suspension culture of *A. belladonna* 48 hours after elicitation with the pellets (P) of different bacterial strains. Bars with the same letter are not significantly different (n=3).

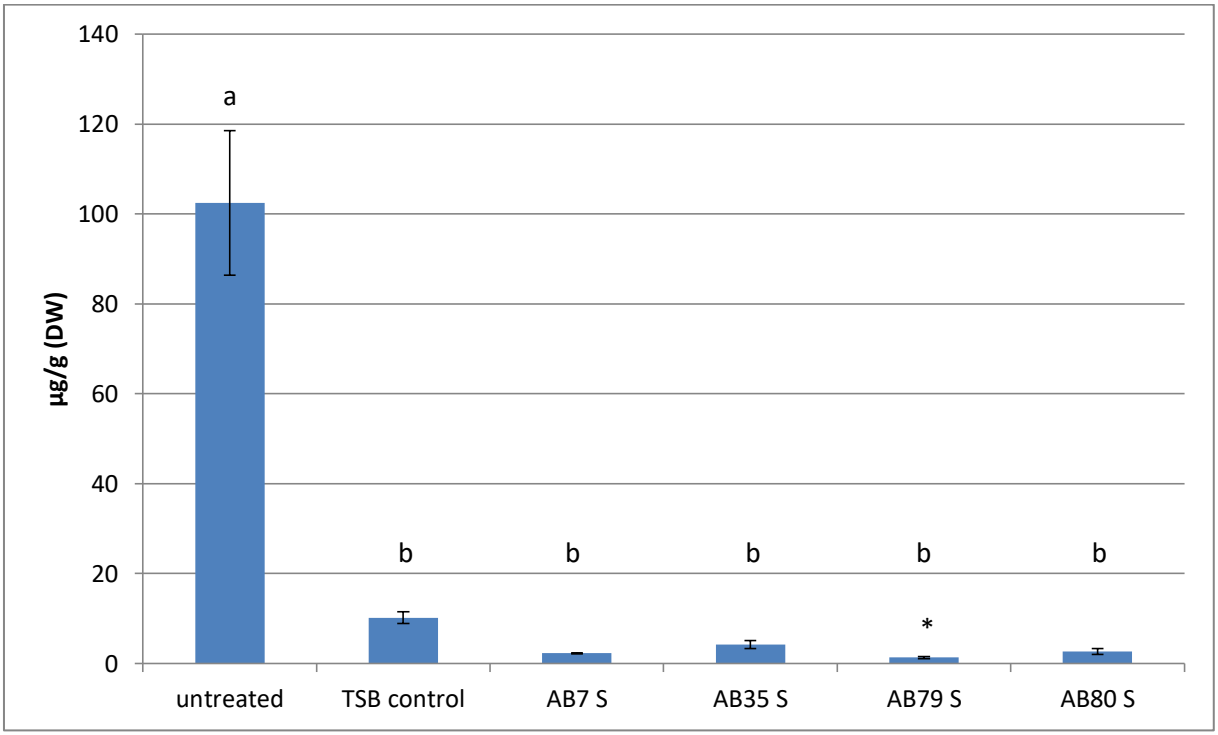

Figure 19: Atropine content of a 7-day-old cell suspension culture of *A. belladonna* 48 hours after elicitation with the supernatant (S) of different bacterial strains. Bars with the same letter are not significantly different (n=3). * Value below LOQ

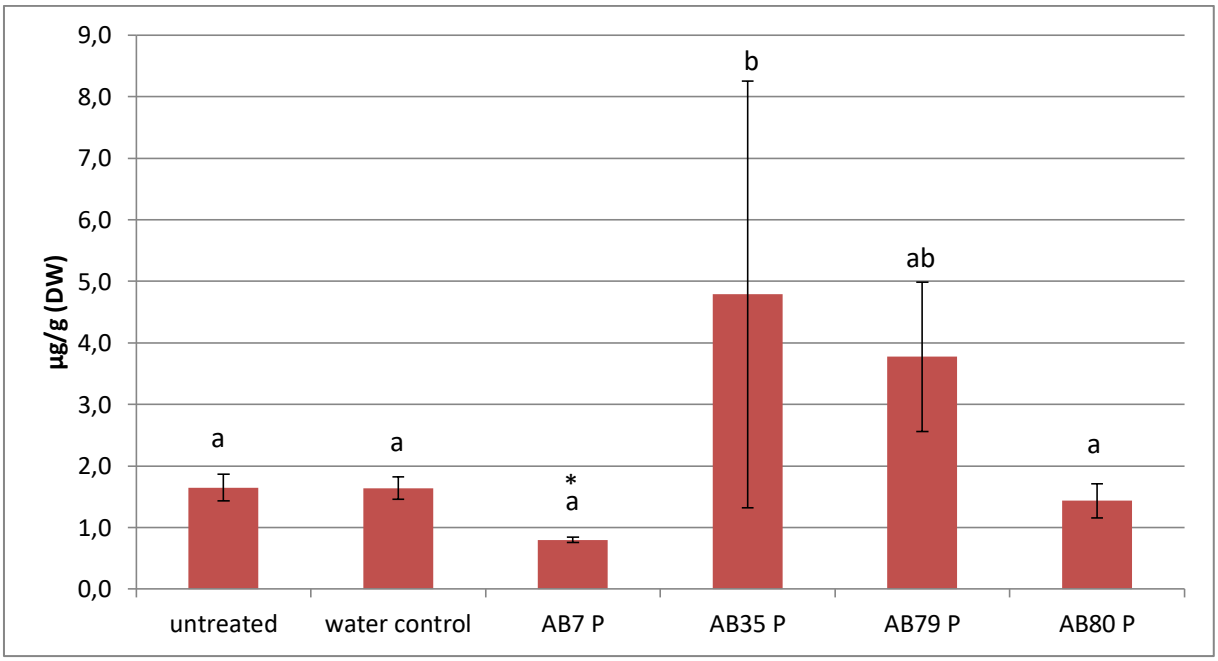

Figure 20: Scopolamine content of a 7-day-old cell suspension culture of *A. belladonna* 48 hours after elicitation with the pellets (P) of different bacterial strains. Bars with the same letter are not significantly different (n=3). * Value below LOQ

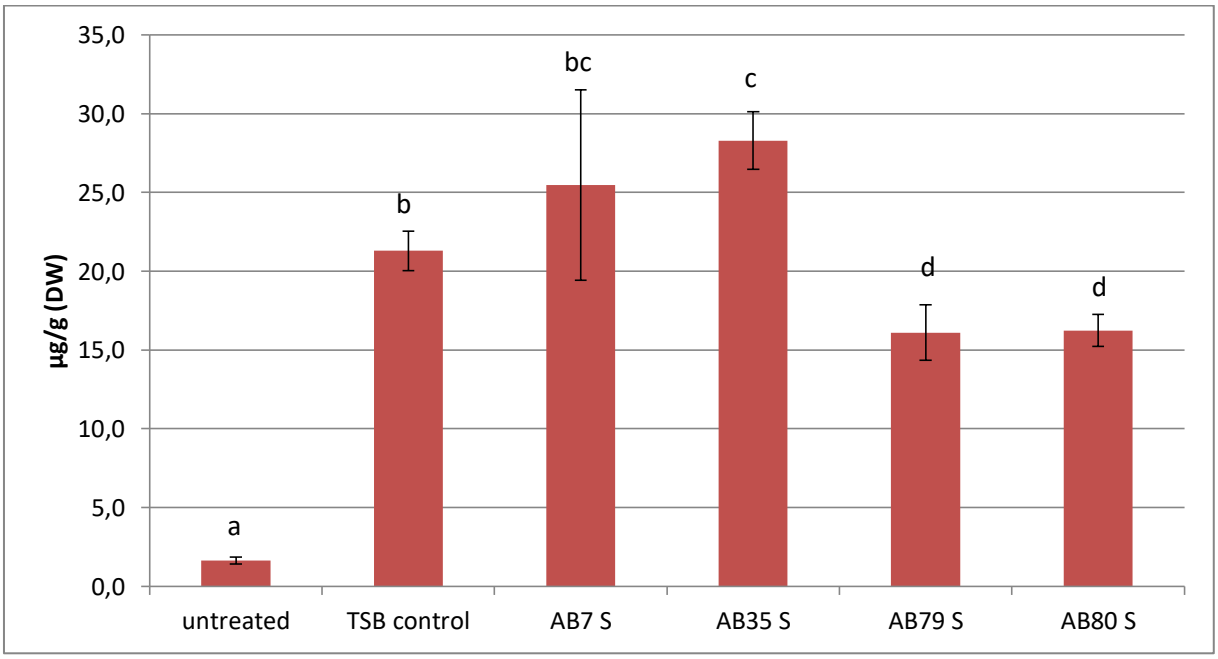

Figure 21: Scopolamine content of a 7-day-old cell suspension culture of *A. belladonna* 48 hours after elicitation with the supernatant (S) of different bacterial strains. Bars with the same letter are not significantly different (n=3).

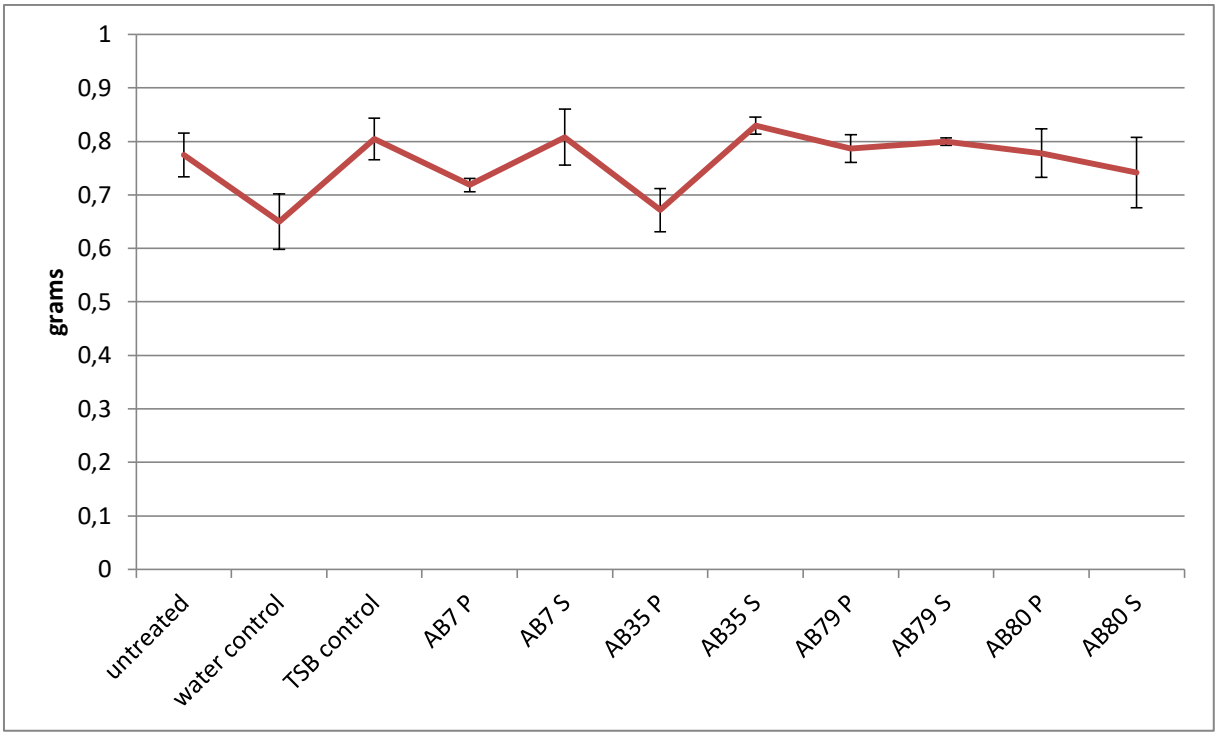

Figure 22: Impact of elicitation after 48 hours with different preparations of bacterial strains on the dry weight of cell suspension cultures of *A. belladonna* (n=3).

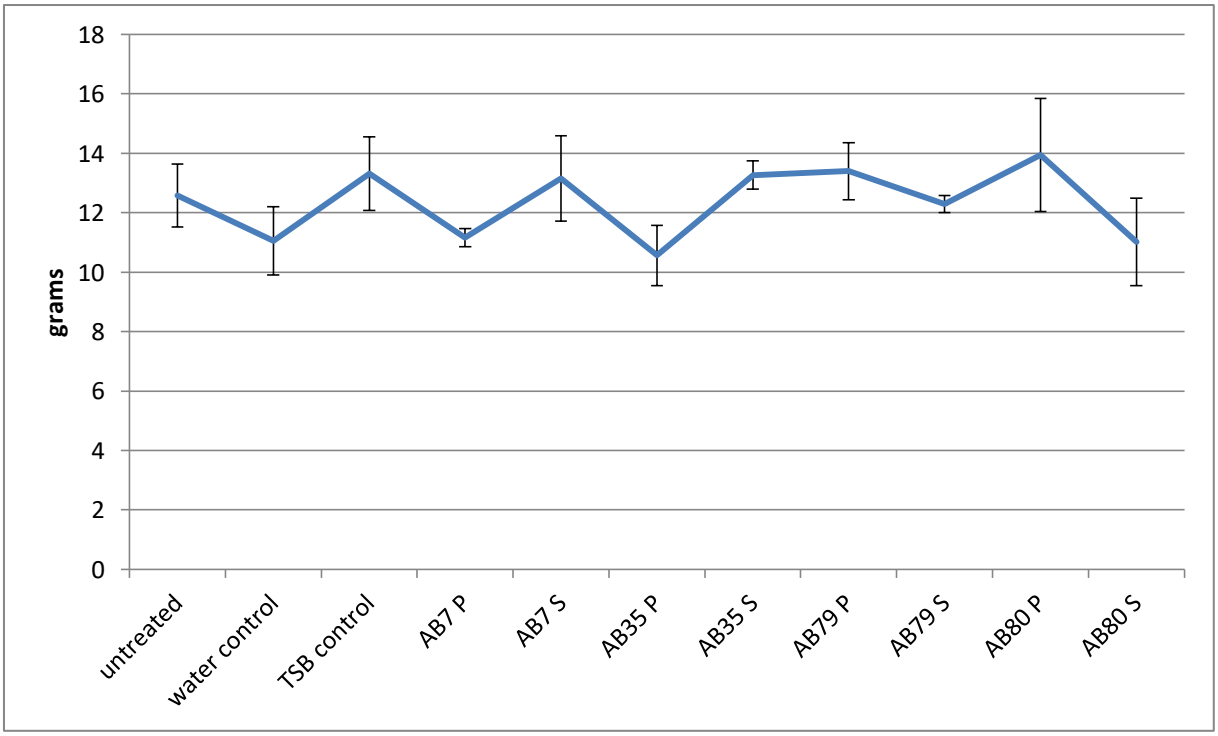

Figure 23: Impact of elicitation after 48 hours with different preparations of bacterial strains on the wet weight of cell suspension cultures of *A. belladonna* (n=3).

6. Discussion

In vitro plant cell and organ cultures represent a promising approach to the production of secondary metabolites, sometimes even at commercial levels. According to Howat et al. (2014) plant cell cultures play an important role in the industrial manufacturing of paclitaxel. Unfortunately, the production of many compounds in significant quantities via plant cell cultures still remains an elusive goal (Ketchum and Gibson, 1996).

There are some limitations to the SM production through plant cell cultures. Working with plant cell cultures starts with the identification of an interesting compound from plant extracts. Challenging is then the collection of plant individuals with high genetic variability. Finding plants with high productivity must be followed by the defining of a suitable culture medium. After cultivating callus cells in suitable medium, a stable production of metabolites has to be reached. Then, a screening of all available cell lines is required to determine their ability to produce acceptable amounts of desired SM (Bourgaud et al., 2001).

After trying out large number of various methods to increase SM formation in plant *in vitro* cultures, it turned out that the most efficient strategy to induce production of metabolites in plants and plant-derived *in vitro* cultures is the use of biotic and abiotic elicitors. Elicitors of biotic origin, such as bacteria represent a good alternative for the enhancement of metabolite generation to traditional methods like modification of the nutrient medium. Using live bacteria compared to autoclaved ones showed promising results in stimulating the biosynthesis of desired compounds through organ cultures but quickly led to damage and death of the plant tissue (Jung et al., 2003). Especially the use of endophytes as biotic elicitors was found to improve production of SM in plants and plant-based *in vitro* cultures. Autoclaved microbes (Ramirez-Estrada et al., 2016) as well as components of endophytes (Ming et al., 2013) could also be used for elicitation.

20 callus cell lines of *A. belladonna* were established prior to this study. One working aim was the establishment of cell suspension cultures out of callus cells, preferably with little aggregate formation and without any organ formation. Basal MS medium supplemented with 10 µM NAA and 2.5 µM kinetin as described by Salem (2018) was used and cell suspension lines of all callus lines were obtained. However, in 12 lines a brownish discoloration was observed. This phenomenon, also known as “tissue browning” is often

caused by oxidation of phenolic compounds, which often results in plant cell death (Khosroushahi et al., 2011). Furthermore, browning goes along with an unwanted transformation of a single cell type to differentiated ones (Tang and Newton, 2004).

The contents of atropine and scopolamine were determined in all cell lines using HPLC-based quantification. Typically, only very low amounts of alkaloids could be detected, which in most cases were below the limits of quantification by the HPLC method. In order to obtain reliable information about the alkaloid production, only one culture per cell line was analyzed as soon as it was available. Thus, due to the known typical variations in biological systems the results of our analyses have to be considered with care.

5 cell lines were subsequently selected based on their alkaloid content, biomass production, aggregate formation and browning. A very important point concerning elicitation is the knowledge of the growth kinetics of the cell suspension culture. According to Bourgaud et al. (2011) the highest amount of SM is produced by cell suspension cultures during the stationary growth phase. The ideal time point for elicitation in order to trigger SM production is, when cultures reach the exponential phase (Chong et al., 2005). Hence, based on the growth characteristics and all other properties (alkaloid content, rapid biomass formation, low aggregation rate) cell line AB 8BW was chosen for elicitation experiments at the exponential phase, actually on day 7 after inoculation.

In 2016, 103 endophyte strains were isolated from *A. belladonna* plants by Dr. Martina Oberhofer (Department of Pharmacognosy, University of Vienna) and the identity of 26 of them could be determined. In this study, four bacterial endophytes belonging to the genera *Pseudomonas*, *Bosea*, *Microbacterium* and *Promicromonospora* were chosen, which had shown promising preliminary results in the studies by Salem (2018).

The preparations of endophytes used for the elicitation in the work at hand were established in two ways. Bacterial cultures grown to the stationary phase in TSB-medium were centrifuged. One elicitor preparation was represented by autoclaved bacterial pellets resuspended in water. The other elicitor was represented by the autoclaved supernatants of bacterial cultures. Interestingly the use of “pellet elicitors” led to the results different from those for the “supernatant elicitors”.

Compared to the untreated control, the 7-day-old cell suspension culture of *A. belladonna* line AB 8BW showed an enhancement in the generation of scopolamine after eliciting them

with the “supernatant elicitors” for 48 hours, but also upon the control treatment with TSB-medium. Most notably were the effects of bacterial sample AB35 S, as it induced an increased scopolamine production in comparison with the TSB-control. The same applied for AB7 S although this effect was not significant. Cultures treated with AB80 S and AB79 S presented a significant enhancement in the scopolamine production compared to the untreated control, but it was less pronounced than in the TSB-treated control.
“Pellet elicitors” had no effect on the scopolamine content except AB35 P which led to a significant increase.

The treatment with “pellet” and “supernatant” elicitors led to a significant decrease of atropine content in the suspension culture. Compared to the TSB-control, the “supernatant” elicitors inhibited atropine formation, but the effect was not significant.

The studies by Salem (2018) also dealt with the impact of autoclaved bacterial endophyte cultures on the alkaloid formation by the cell suspension cultures of *A. belladonna*. Bacterial strains were cultured in TSB medium as in this work. Compared to this study, Salem (2018) did not separate into “supernatant” and “pellet” elicitors after cultivation of bacterial strains. Among other bacterial strains, he also worked with AB 35 (*Pseudomonas sp.*), AB 7 (*Promicromonospora sp.*), AB 80 (*Bosea sp.*) and AB 79 (*Microbacterium sp.*). Only the supernatants of bacterial cultures autoclaved as a whole were used as elicitors. Nevertheless, the results were similar to those in this work. These four strains used in both studies led to a significant decrease of the atropine production compared to the zero-controls. For the most part in this work, the elicitors, including TSB medium control led to a significant enhancement of the scopolamine content compared to the zero-controls. The work of Salem (2018) also showed an increase of the scopolamine content in the culture AB 35 compared to the zero-control. In both works, the strain AB 7 led to a significant increase of the scopolamine content compared to the zero-controls. However, the cell suspension line used by Salem had a distinctly lower alkaloid content and all HPLC analyses led to values below the LOQ.

The study by Jung et al. (2003) showed as well, that some bacteria used as biotic elicitors induced a significant increase only of the scopolamine content of hairy root cultures of

Scopolia parviflora, which, like *A. belladonna,* is also a member of the family Solanaceae. It was also demonstrated that the atropine content was not increased by those elicitors. In addition to the usage of autoclaved bacteria as elicitors, plant *in vitro* cultures were also treated with live bacteria in contrast to this present work, which only dealt with autoclaved ones. In the work by Jung et al. (2003), autoclaved bacterial elicitors showed no effects on the alkaloid formation.

Jung et al. (2003) also investigated the expression level of the enzymes hyoscyamine 6β-hydroxylase (H6H) and putrescine N-methyltransferase (PMT). H6H is responsible for the conversion of hyoscyamine to scopolamine and PMT induces the biosynthesis of the first tropane alkaloid precursor, N-methylputrescine. Jung et al. (2003) observed that upon treatment with bacterial elicitors the ratio of scopolamine to hyoscyamine increased as well as the expression level of PMT. However, despite of the increase of the scopolamine to hyoscyamine ratio, the expression of H6H decreased. The authors stated that the biosynthesis of tropane alkaloids is not yet fully understood.

Another investigation demonstrated the effects of a biotic, living elicitor, *Aspergillus niger*, on the alkaloid formation in adventitious root cultures of *Datura metel*, also a Solanaceous plant. The homogenate of mycelia of *A. niger* washed with distilled water led to an accumulation of tropane alkaloids (Ajungla et al., 2009). Shakeran et al. (2017) demonstrated the impacts of biotic elicitors on the alkaloid content in hairy root cultures of *Datura metel*. They used live bacterial cultures of *Bacillus cereus* and *Staphylococcus aureus* and could observe an increased production of scopolamine and a decrease in atropine content (Shakeran et al., 2017).

Typically, the response of an *in vitro* culture to elicitation can extend over a considerable time span, and the concentration of SM of interest can vary as well. In this work, we analyzed the alkaloid content 48 hours after elicitation. Jung et al. (2003) monitored atropine and scopolamine formation 12, 24 and 48 hours after treatment and found considerable variations. While upon elicitation with *Staphylococcus aureus* scopolamine contents after 12 and 24 hours were significantly higher than in the control, no scopolamine was detectable anymore after 48 hours. Additional future investigations could similarly monitor alkaloid concentrations in cell suspension cultures of *A. belladonna* at different time points after elicitation.

Finally, one parameter which was not evaluated in this study was the amount of elicitor used for the treatments. Many authors have described a correlation between elicitor concentration and SM production. Wawrosch et al. (2014) for example demonstrated that some elicitors had a dose dependent effect on the formation of lignans in hairy root cultures of *Leontopodium nivale*. In continuation of the present study with cell suspension cultures of *A. belladonna* such investigations may also be suggested

7. References

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