PALACKY UNIVERSITY OLOMOUC

Faculty of Natural Sciences

Department of Biochemistry

GENETIC MODIFICATION OF *CLAVICEPS PURPUREA* AND ERGOT ALKALOIDS PRODUCTION

Ph.D. Thesis

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I hereby declare that this thesis has been written solely by myself and that all the sources used in this thesis are cited and included in references part. All results included in this work are approved by co-authors.

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In Olomouc Helena Hanosová

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Bibliografická identifikace

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Jazyk	Anglický

Abstrakt

Předkládaná disertační práce je zaměřená na studium a ovlivňování produkce námelových alkaloidů organismem *Claviceps purpurea* a sestává ze tří částí.

V první části disertační práce je vypracována odborná rešerše, zabývající se biologií organismu *Claviceps purpurea*, genetikou produkce námelových alkaloidů, jejich chemickou strukturou a z ní vyplývající biologickou aktivitou. Jsou popsány metody průmyslové produkce námelových alkaloidů, metody genové transformace organismu *C. purpurea* a možnosti ovlivnění produkce námelových alkaloidů metodami molekulární biologie.

Druhá část disertační práce je zaměřená na optimalizaci polní produkce námelových alkaloidů aplikací vhodného gametocidu. Výnos sklerocií může být významně redukován pylem pocházejícím z nedokonale pylově sterilního žita, jelikož pouze neoplodněná ovaria mohou být sporami *C. purpurea* infikována. Aplikací vhodného gametocidu dojde k významnému zlepšení sterility žita s nedokonale navozenou cytoplasmatickou sterilitou či indukci sterility u fertilního žita. Maleinhydrazid prokázal vysokou míru gametocidního účinku u obou variant žita, přičemž měl zanedbatelný efekt na klíčení spor *C. purpurea*. Optimální čas aplikace (2-3 týdny před antézou) i optimální dávkování byly klíčové pro zisk vysokého množství sklerocií při minimálním znečištění zrnem.

Třetí část disertační práce je zaměřena na genetiku produkce ergopeptidů v průmyslových kmenech *C. purpurea*. Na oddělení Molekulární biologie, Centra regionu Haná, Palackého Univerzity byla za laskavé pomoci skupiny Prof. Tudzynského zavedena optimalizovaná metoda transformace *C. purpurea*, zajišťující zisk velkého množství transformantů za transformaci. Metoda byla použita k přípravě dvou nezávislých *LpsA2* knock-out mutantů vyselektovaných z množství 230 transformovaných kolonií. Chemická analýza knock-out mutantů stejně jako vzorků s náhodně integrovanou deleční kazetou prokázala degeneraci kmene a z ní vyplývající neschopnost produkce námelových alkaloidů, znemožňující vyhodnocení experiment.

Tři geny klastru pro biosyntézu námelových alkaloidů (tj. *dmaW, easC* a *easG*) byly vybrány jako potenciální limitující kroky dráhy, určující míru produkce námelových alkaloidů. Jejich exprese pod silným konstitutivním promotorem gpdA nezapříčinila významné a udržitelné zvýšení produkce námelových alkaloidů v testovaných kmenech.

Gen *LpsA1,* odpovědný za produkci ergotaminu v kmeni 20.1 byl konstitutivně exprimován v kmeni Gal 130, produkujícím ergokristin jako hlavní alkaloid. Dva nezávislí Gal 130: OE*LpsA1*, 20.1 transformanti byli testováni pro kvalitativní změny v produkci námelových alkaloidů. Bylo u nich zjištěno významné zvýšení relativního zastoupení ergotaminu, jež je ve WT kmeni Gal 130 produkován v míře přibližně 1% z celkového obsahu námelových alkaloidů.

Bibliografic identification

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Abstract

Presented thesis is focused on study of ergot alkaloids and on affecting of their production in *Claviceps purpurea* and is divided into three parts.

The first part of the thesis is devoted to the literature review focused on biology of the *Claviceps purpurea*, genetics of ergot alkaloids production, their chemical structure and resulting biological activity. Methods of industrial production of ergot alkaloids, methods of genetic transformation of the *Claviceps purpurea* and possibilities for affecting of the ergot alkaloids production by means of molecular biology techniques are described.

The second part of the thesis is focused on optimalization of the field production of ergot alkaloids by the gametocide application. Yield of sclerotia can be significantly reduced by presence of pollen contamination originating from imperfect male-sterile rye, as only unfertilized ovaria can be infected by spores of *Claviceps purpurea*. Application of suitable gametocide leads to significant improvement of rye male-sterility of both, a fertile rye variety and a variety with imperfectly induced cytoplasmic male sterility. Maleic hydrazide was proven to be a highly effective gametocide for both of the above-mentioned variety of rye and showed

negligible effect on germination of *C. purpurea* spores. Both accurate dosaging of the active gametocidal compound and timing of the application just 2–3 weeks before onset of anthesis proved crucial to achieving high ergot yield with minimum grain impurities.

The third part of the thesis is focused on genetic background of the production of ergopeptides in industrial strains of *C. purpurea*. Optimized method for transformation of *C. purpurea* was introduced at the department of Molecular Biology, Centre of region Haná with kind help of Prof. Tudzynski´s group and provides large number of transformants per transformation. The method was used for generation of two independent *LpsA2* knock-out mutants, selected from total of 230 transformants. Chemical analysis of the knock-out mutants showed degeneration of the strain, resulting in loss of the ability to produce ergot alkaloids in the knockout mutants, as well as in the transformants with an ectopically integrated knockout cassette. Degeneration of the strain made evaluation of experiment impossible.

Three genes of cluster for biosynthesis of ergot alkaloids (ie. *dmaW, easC* and *easG*) were selected as potential limiting steps of the pathway, determining the rate of the production of ergot alkaloids. Expression of selected genes under the control of strong, constitutive promoter gpdA didn´t lead to any biotechnologically significant, sustainable changes in EA production of tested strains.

The *LpsA1* gene, responsible for the production of ergotamine in the 20.1 strain, was constitutively expressed in the Gal 130 strain, the ergocristine producer. Two independent Gal 130:OE*LpsA1*, 20.1 transformants were analyzed for qualitative changes in their ergot alkaloid production. A significant improvement in ergotamine representation, which is originally the minor alkaloid of the strain, representing in WT only around 1% of the EA pool, was observed in both of the mutants.

TABLE OF CONTENT

Objectives

1. Elaboration of scientific review on the *Claviceps purpurea* and ergot alkaloids production

2. Introduction of effective method of transformation of *Claviceps purpurea* industrial strains at Department of Molecular Biology, Center of Region Haná, Palacký University

3. Constitutive, strong expression of selected genes of the pathway of biosyntheses of ergot alkaloids and testing of its impact on quality and quantity of produced alkaloids

4. Optimalization of method of field production of ergot alkaloids

Part I – General Introduction - Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids

1.1 Abstract

Ergot alkaloids produced by the fungus *Claviceps* parasitizing on cereals, include three major groups: clavine alkaloids, D-lysergic acid and its derivatives and ergopeptines. These alkaloids are important substances for the pharmatech industry, where they are used for production of anti-migraine drugs, uterotonics, prolactin inhibitors, anti-Parkinson agents, etc. Production of ergot alkaloids is based either on traditional field cultivation of ergot-infected rye or on submerged cultures of the fungus in industrial fermentation plants. In 2010, the total production of these alkaloids in the world was about 20,000 kg, of which field cultivation contributed about 50%. This review covers the recent advances in understanding of the genetics and regulation of biosynthesis of ergot alkaloids, focusing on possible applications of the new knowledge to improve the production yield.

1.2 Introduction

The *Clavicipitaceae* family includes, among others, a fungal species called ergot that is important for humankind, in terms of both contributions and losses. In medieval history, these fungi parasitizing in cereals and producing a range of ergot alkaloids caused many mass poisonings that resulted in painful deaths of tens of thousands of people (Schiff, 2006). Nowadays, mass poisonings with ergot alkaloids are sporadic and occur only in developing countries. For example, an epidemic of ergotism with a high mortality rate occurred in 1977 in Ethiopia (Demeke et al., 1979). Contrary to mass poisonings, the recent cases of individual poisonings are usually connected with overdoses of medical drugs based on these alkaloids.

For thousands of years, people tried to take advantage of substances produced by ergot without knowing their chemical structure, proper dosage or side effects, but scientific research on ergot alkaloids started only with the beginning of modern pharmacy in the early 20th century. Nowadays, specific types of ergot alkaloids are widely used as a basic drug-stock for the production of various therapeutic substances, e.g. for the treatment of migraine, in gynecology for its uterotonic effects, as prolactin inhibitors, antiparkinsonian drugs, etc. (de Groot et al., 1998).

The *Claviceps* genus is not only interesting for its ability to produce secondary metabolites usable in pharmaceutical industry, but also in its life strategy, mainly the specificity of invaded host organs, which is in the center of interest of many scientific groups. Last, but not least, its negative influence in agriculture should be mentioned. Some of the *Claviceps* species cause significant losses in cereal production. Recently, there was an infection of the fifth most important cereal crop in the world, *Sorghum bicolor* (Haarmann et al., 2009), by *Claviceps africana*.

1.3 Ergot alkaloids

1.3.1. History of poisoning and use of ergot alkaloids

First references to ergot use can be found in early history. The oldest documentation of the positive effects of ergot alkaloids in obstetrics appeared in China in approximately 1100 BC (Schiff, 2006). In one of the sacred books of the Parsees (400 BC to 300 BC), ergot is mentioned as “noxious grasses that cause pregnant women to drop the womb and die in childbirth” (Thoms, 1931). In the Middle Ages, there were many documented cases of mass intoxication with ergot caused by contaminated cereals, usually referred to as ergotism. There are two types of ergotism, which differ in symptoms. The first one, called “convulsive ergotism”, was typical for the area located east of the Rhine river in Europe and was accompanied by muscle spasms, hallucinations and fever. These symptoms are typical for serotonergic stimulation of the central nervous system, caused by the activation of serotonin receptors by ergot alkaloids, due to their structural similarity to the neurotransmitter serotonin (Eadie, 2003). The second type of ergotism, typical for the area west of Rhine, is called “gangrenous ergotism”, and is accompanied by violent burning and shooting pain of the affected acral part of the human body. Related to the saint who suffered horrible visions sent by the devil, this type of ergotism has been called St. Anthony's fire (Lee, 2009). The first documented epidemic of ergotism is dated 944–945 AD and caused the death of about 10,000 people in France. Some 50 years later, intoxication with ergot alkaloids again killed about 40,000 people in this area. It is likely that ergot alkaloid intoxication was also connected with the well-known witch trials of 1692 in Salem, Massachusetts, USA (Caporael, 1976; Spanos and Gottlieb, 1976) and in Finnmark,

Norway in the 17th century (Alm, 2003). A correlation between the symptoms of ergotism and ergot consumption was understood finally in the 1850s, due to the findings of Louis René Tulasne, a French mycologist, who first fully described the life cycle of ergot (Tulasne, 1853). Modern ergot alkaloid research started in 1918 with ergotamine isolation by the Swiss biochemist Arthur Stoll (Stoll, 1945). In 1926, Swiss psychiatrist Hans Maier suggested that ergotamine might be useful in the treatment of vascular headaches of the migraine type (Silberstein et al., 2001). LSD, a synthetic derivative of lysergic acid, is one of the components of ergot alkaloid blend that was first synthesized in 1938 by the Swiss chemist, Alfred Hoffman, but its effect on nervous system was not discovered until he accidentally contaminated himself and experienced the hallucinogenic reaction in 1943 (Minghetti and Crespi-Perellino, 1999). The drug became popular in the mid-1960s when its sensealtering properties were reputed to offer a window into enhanced creativity and self-awareness.

1.3.2. Chemistry and occurrence of ergot alkaloids

Ergot alkaloids belong to the class of indole derivatives. They can be divided into three major groups: clavine alkaloids, D-lysergic acid and its simple derivatives and ergopeptines. Structures of typical ergot alkaloids are shown in Fig. 1. Species within the genus *Claviceps* differ in their capability to produce diverse types of alkaloids. Only a few species (e.g. *Claviceps purpurea* and *C. africana*) can produce ergopeptines as final products of their ergot alkaloid biosynthetic pathway. For example, the biosynthetic pathway of *Claviceps fusiformis* ends with elymoclavine production that has been explained as a loss of the late pathway genes in the ergot alkaloid gene cluster (Lorenz et al., 2007).

Most of ergot alkaloids contain a tetracyclic ergoline structure, although some of the naturally occurring clavine alkaloids are tricyclic, e.g. chanoclavine-I, chanoclavine-II and isochanoclavine-I (for a review see Buchta and Cvak, 1999). Of these, only chanoclavine-I can serve as a precursor for biosynthesis of other ergot alkaloids. Tetracyclic clavine alkaloids can be found in various species of fungi. Agroclavine and elymoclavine are intermediates of the pathway leading to D-lysergic acid production, but in most ergot alkaloid producing fungi, this pathway ends with clavine alkaloids as final products (see Fig. 2A). Besides the genus *Claviceps*, ergot alkaloids were also once reported in other fungi, such as *Botrytis fabae* (Naim, 1980) and *Geotrichum*

candidum (El-Refai et al., 1970). However, their occurrence was later unconfirmed by more advanced analytical methods. Similarly, ergopeptines were mentioned to be produced by *Aspergilus fumigatus* (Abe et al., 1967; Cole et al., 1977; Narayan and Rao, 1982; Spilbury and Wilkinson, 1961), but this was not confirmed more recently.

Derivatives of D-lysergic acid can be divided into simple amides and tripeptides. One of the representatives of D-lysergic amides is ergometrine (alternatively also called ergonovine or ergobasine) derived from D-lysergic acid and 2-aminopropanol. Ergometrine is produced by *C. purpurea* together with ergopeptines (Dudley and Moir, 1935), which are built from the lysergic acid fragment and a tripeptide group composed of diverse amino acids, where proline is always in the third position. Ergopeptines are the end products of the biosynthetic pathway of ergot alkaloids. The main producer of ergopeptines is *C. purpurea*, but dihydro-α-ergosine is also produced by *C. africana* (Mantle and Waight, 1968).

The presence of clavine alkaloids and ergopeptines had been for a long time also associated with dicotyledonous plant species of the family *Convolvulaceae*, also known as the bindweed or morning glory. In these plants, the alkaloids are produced by different species of endophytic fungi that are mostly seed-transmitted and may have shared their alkaloid producing genes by hybridization or horizontal transfer (Clay and Schardl, 2002). Microscopic clavicipitalean fungi were recently detected residing on leaves and seeds of *Ipomoea asarifolia* and *Turbina corymbosa* (Steiner et al., 2006). This epibiotic fungi that contains genes involved in ergot alkaloid biosynthesis (Markert et al., 2008) was recently described as a new genus, *Periglandula* (Steiner et al., 2011).

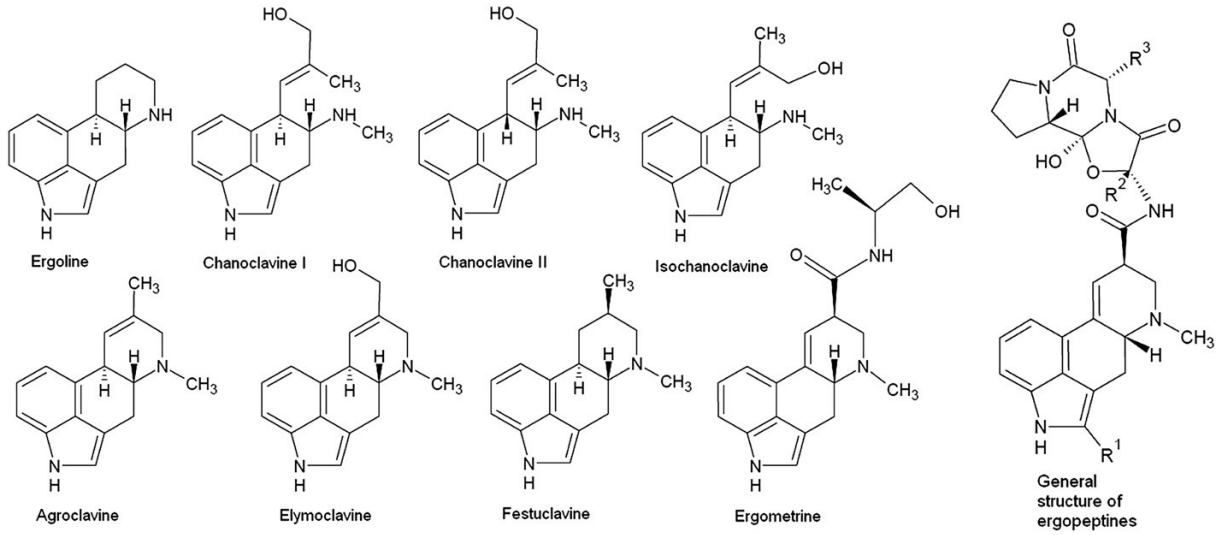


Figure 1. Structures of ergot alkaloids.

1.3.3. Biosynthesis of ergot alkaloids: enzymology and genetics

Essential steps of the ergot alkaloid biosynthetic pathway were analyzed by feeding *Claviceps* liquid cultures with isotopically labeled precursors. The general biosynthetic pathway of ergot alkaloids is shown in Fig. 2A,B. As precursors of the ergoline system, three primary metabolites were found: tryptophan, a product of the mevalonate pathway dimethylallylpyrophosphate (DMAPP) as a donor of the isoprene unit, and methionine as a methyl group donor. The first specific step of the biosynthetic pathway is the isoprenylation of tryptophan resulting in 4-(γ,γ-dimethylallyl)tryptophan (DMAT). This step, catalyzed by 105 kDa homodimer enzyme DMAT synthase (Gebler and Poulter, 1992), is positively regulated by tryptophan and negatively feedback-regulated by agroclavine and elymoclavine, further products of the biosynthetic pathway (Cheng et al., 1980). *DmaW* gene coding for DMAT synthase, originally cloned and sequenced from *C. fusiformis*, was the first discovered gene of the ergot alkaloid synthesis (EAS) cluster (Tsai et al., 1995). The 1517 bp ortholog of *dmaW* containing two introns was found in *C. purpurea*, proving a common biosynthetic origin of clavines and D-lysergic acid-derived alkaloids (Arntz and Tudzynski, 1997). Analyses of 3′ flanking region of *dmaW* showed a presence of another EAS related gene named *cpps1* (Tudzynski et al., 1999). By chromosome walking, a whole 68.5 kb EAS cluster, consisting of 14 genes (Fig. 2C), was described by Paul Tudzynski's group (Haarmann et al., 2005).

The second step of the EAS pathway is the methylation of DMAT to *N*-methyl-dimethylallyltryptophan (MeDMAT). Otsuka et al. (1980) first isolated an enzyme that is responsible for catalyzing the methylation of DMAT using S-adenosylmethionine as a donor of the methyl group. The biosynthetic pathway continues with several oxidation and reduction steps resulting in the formation of chanoclavine I, chanoclavine I aldehyde and then agroclavine. Chanoclavine I is the only one of the four stereoisomers of chanoclavine, which can be further converted into tetracyclic ergolines (Gröger et al., 1966). Chanoclavine I synthase, a FAD-containing oxidoreductase, is involved in the oxidation of MeDMAT to chanoclavine I, but there are still some other enzymes needed to complete the conversion. The mechanism of this reaction was proposed by Gröger and Floss (1998). In *C. purpurea*, chanoclavine synthase is encoded by *ccsA* gene of 1503 bp, originally named *easE*. The coding region of *ccsA* gene is interrupted by one intron (Lorenz et al., 2010). Chanoclavine-I is then further oxidized to chanoclavine-I aldehyde by an alcohol dehydrogenase encoded by *easD* (Wallwey and Li, 2011).

Closure of D ring of tetracyclic ergot alkaloids proceeds via *cis-trans* isomerization on C2 of the mevalonate group (Floss, 2006) in a series of enzymatic steps, collectively called chanoclavine cyclase. Chanoclavine-I aldehyde first undergoes double bond isomerization to isochanoclavine-I aldehyde, originally thought to be catalyzed by the product of *easA* gene. It was shown recently that the reaction also proceeds non-enzymatically by reduction with glutathione or 2- mercaptoethanol (Matuschek et al., 2011). Isochanoclavine-I aldehyde then forms an iminium ion compound, which is subsequently reduced to agroclavine by the enzyme easG (agroclavine synthase) in the presence of NADPH. The monomeric easG of 31.9 kDa consists of 290 amino acids and is encoded by *easG* gene of 1079 bp containing two introns (Matuschek et al., 2011).

The biosynthetic pathway then continues with a cloA catalyzed oxidation of agroclavine on C17 atom, leading to formation of elymoclavine. Elymoclavine is oxidized to paspalic acid again by clavine oxidase cloA (Haarmann et al., 2006; Robinson and Panaccione, 2014). This enzyme connects the formation of two groups of ergot alkaloids, the clavine alkaloids and D-lysergic acid derived alkaloids, such as simple D-lysergic acid amides and ergopeptines. CloA protein is encoded by *cloA* gene of 2120 bp interrupted with eight introns (Haarmann et al., 2006). Paspalic acid is converted to D-lysergic acid spontaneously, as described by Gröger

and Floss (1998).

Elymoclavine monooxygenase and all subsequent enzymes are not encoded in fungal genomes that produce the clavine type of alkaloids. The following metabolic steps lead to amides of D-lysergic acid and ergopeptines, crucial metabolites of *C. purpurea*. D-lysergic acid is captured and activated by monomodular non-ribosomal peptide synthetase (NRPS) LPS2, one of the two subunits of D-lysergyl peptide synthetase with a molecular mass of 141 kDa. D-lysergic acid is, after binding to LPS2 as a thioester, transferred to trimodular LPS1 also named LPSA1 NRPS, the second subunit of D-lysergyl peptide synthetase with a size of 370 kDa. Here it is condensed with three bound amino acids, resulting in formation of a D-lysergyl tripeptide lactam (Walzel et al., 1997). LPS2 and LPS1 NRPS are encoded by *cpps2* and *cpps1* genes, respectively (Correia et al., 2003; Tudzynski et al., 1999). *Cpps2*, also named *lpsB*, is a gene of 3991 bp containing one intron, while *cpps1*, also named *lpsA1*, consists of 10,850 bp and contains two introns.

Finally, D-lysergyl peptide lactam is oxidized by easH (Havemann et al., 2014) and the product is spontaneously converted to ergopeptines (Haarmann et al., 2006). The structure of final ergopeptine depends on the amino acids used for the condensation with D-lysergic acid, where the first two positions are occupied by non-polar amino acids and the third one exclusively by proline (Keller, 1999).

C. purpurea can also synthesize D-lysergylalkanolamides, alkylamides of D-lysergic acid, such as ergometrine. In these compounds, D-lysergic acid is linked to an aminoalcohol derived from alanine. The reaction is catalyzed by LPS2 as the D-lysergic acid activator and another monomodular NRPS subunit, ergometrine synthetase, in the presence of NADPH. LPS3 enzyme (ergometrine synthetase) is encoded by *cpps3* gene (also called *lps3* or *lpsC*) located in the EAS gene cluster (Ortel and Keller, 2009).

The EAS cluster contains one more NRPS gene, *cpps4* (also called *lps4* or *lpsA2*), which encodes LPS4 protein also named LPSA2 with a function similar to LPS1. The two enzymes differ in their capability to bind different amino acids used for ergopeptine formation. LPS4 is responsible for α-ergocryptine production in P1 strain of *C. purpurea*, while LPS1 catalyzes the production of ergotamine (Haarmann et al., 2005).

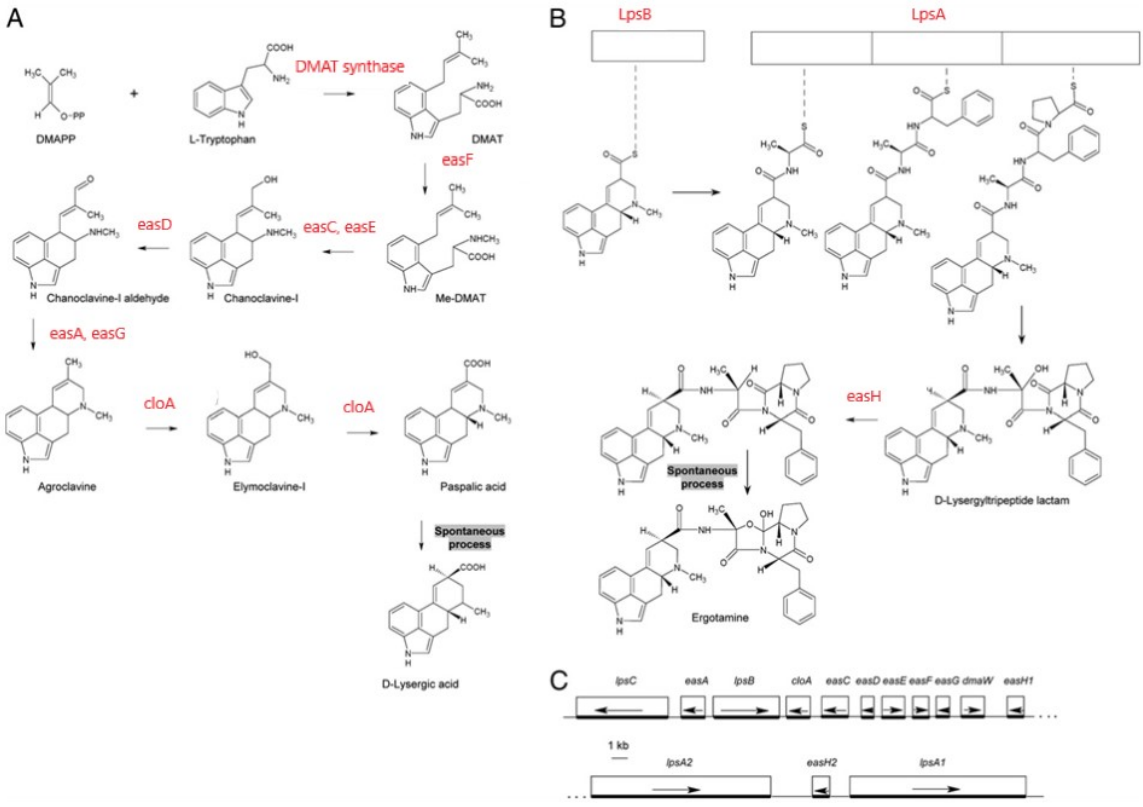


Figure 2. Biosynthesis of ergot alkaloids in *Claviceps purpurea*: (A) scheme of the biosynthetic pathway leading to D-lysergic acid, (B) biosynthesis of ergopeptines on two-component non-ribosomal peptide synthetase (NRPS), and (C) scheme of the ergot alkaloid synthesis (EAS) gene cluster.

1.3.4. Biological activity of ergot alkaloids

Specific types of ergot alkaloids serve as a basic drug-stock for production of various therapeutic substances to treat migraine headaches, Parkinson's disease, hypertension and diverse sexual disorders (de Groot et al., 1998). Biological activity of ergot alkaloids relates to their structural similarities with neurotransmitters noradrenalin, dopamine and serotonin (Fig. 3). The major pharmaceutical effects of ergot alkaloids are smooth muscle stimulation, central sympatholytic activity and peripheral α1-adrenergic blockade. Smooth muscle stimulation is the most evident as a vasoconstriction and uterine contraction (Innes, 1962). Since the ergot alkaloids are dopamine receptor agonists, some of them (bromocriptine, cabergoline, pergolide) can be used as inhibitors of prolactin release (Nasr and Pearson, 1975) and anti-Parkinson agents. However, they are not recommended as a first-line

antiparkinsonian medication because of the risk of fibrotic reaction (Bonuccelli et al., 2009).

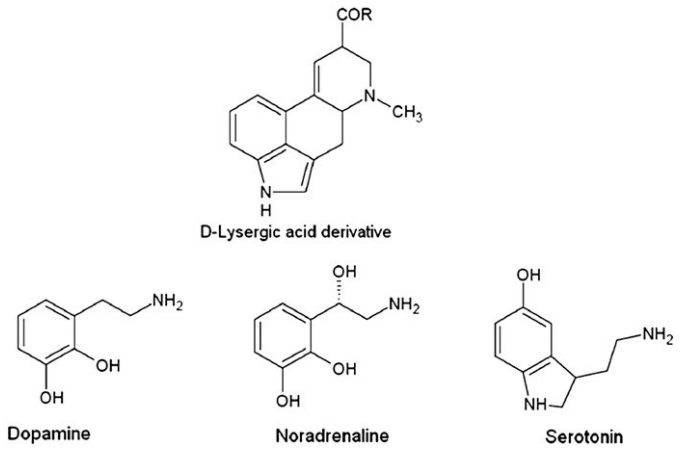


Figure 3. Structural similarities between the derivatives of D-lysergic acid and neurotransmitters dopamine, noradrenalin and serotonin.

Effects of the ergot alkaloids on dopamine receptors are described in more detail in Emilien et al. (1999). Anti-migraine effects of many ergot alkaloids are facilitated by interactions with serotonin receptors, 5-HTs. First compound used for the treatment of this disorder was ergotamine in 1926 (Maier, 1926). The main disadvantage of natural ergot alkaloids is a lack of selectivity for each individual 5-HT receptor, which stimulated the development of semisynthetic serotonergic ligands that are more selective (Pertz and Eich, 1999). Interactions of the ergot alkaloids and their derivatives with serotonin receptors are reviewed in Pertz and Eich (1999). Ergot alkaloids also interact with α_1-adrenoceptors, which mediate vascular contractility. Many ergot alkaloids and their derivatives are partial antagonists or agonists of these receptors, which may be the cause of their cardiovascular side effects. The effect of ergot alkaloids on α_1 receptors is described in Görnemann et al. (2008). Agroclavine was also mentioned to show antibacterial effects (Schwarz and Eich, 1983) and cytostatic effects in LSI78Y mouse lymphoma cells (Glatt et al., 1987), but these finding are not supported by more recent studies.

1.4 Production of ergot alkaloids for pharmaceutical industry

1.4.1 The fungus *Claviceps*

There are 36 members of genus *Claviceps*, which can infect about 600 species of monocotyledonous plants. The *Claviceps* genus includes some economically important species because they infect agronomically valuable plants (Bové, 1970). A prominent member, *C. purpurea*, is spread worldwide and has the widest host range of any *Claviceps* species. It can infect about 400 species of grasses (Taber, 1985), but its most typical hosts are rye, wheat and barley (Loveless, 1971). *Claviceps africana*, *Claviceps sorghi* and *Claviceps sorghicola* infect *S. bicolor* (Haarmann et al., 2009) grown in eastern and southern Africa, Southeast Asia, Japan, South America and Australia, *Claviceps gigantea* is found on *Zea mays* in central Mexico, *Claviceps paspali* on *Paspalum* species and *C. fusiformis* on *Pennisetum americanum* (Pažoutová and Parbery, 1999). In all *Claviceps* species, the infection is strictly targeted to the host ovaries (Parbery, 1996).

C. purpurea exhibits a life cycle shown in Fig. 4, which is typical for the *Claviceps* species.

Figure 4. Life cycle of the fungus *Claviceps purpurea*: 1) opened rye floret, 2) hyphae invasion of the rye ovary, 3) honeydew production, 4) reinfection by an insect, 5) mature sclerotia formation, 6) overwintering of sclerotia, 7) germinating sclerotia, 8) release of ascospores.

The sexual life cycle starts with an infection of unfertilized rye ovaries (Fig. 5A). Specialized penetration structures are not known in *C. purpurea*, indicating that the cell wall is passed due to enzyme secretion (Haarmann et al., 2009). Conidia are transported to the plant by wind or insects, the plant cuticle is penetrated and fungal hyphae colonize ovarian tissues. After 5 to 7 days post infection, the first macroscopically visible sign of infection, production of honeydew, can be detected (Fig. 5B). This liquid contains plenty of sugars attractive for insects and is filled with conidia, which enables secondary infection (Swan and Mantle, 1991; Tenberge,

1999; Tulasne, 1853). *C. purpurea* honeydew also contains inhibitors of conidial germination of many other fungi (Cunfer, 1976). The honeydew production stops after about 2 weeks being followed by the development of a rigid stage sclerotium, composed of a compact mass of hardened fungal mycelium (Tenberge, 1999; Tudzynski and Scheffer, 2004) as shown in Fig. 5C. After 5 weeks post infection, sclerotia mature (Kirchhoff, 1929). Sclerotia can survive unfavorable conditions in winter and germinate after a temperature increase in the spring (optimally at about 20 °C) on or beneath the soil surface (Kirchhoff, 1929), forming stromata composed of stalks with spherical capitula that grow in a phototropic manner to reach the air (Hadley, 1968). In nature, sclerotia are the unique structures, which produce all types of ergot alkaloids (Ramstad and Gjerstad, 1955).

1.4.2. Field production on rye

The fungus *C. purpurea* is a species widely used in the pharmaceutical industry for its ability to produce ergot alkaloids. Clavine alkaloids, being derivatives of tetracyclic ergoline ring structure, are produced by various types of fungi. On the other hand, ergopeptines, peptidyl derivatives of D-lysergic acid amides, are produced only by some members of *Clavicipitaceae* family and their preparation by organic synthesis is economically unprofitable. The world production of ergot alkaloids reaches thousands of kilograms annually that include both ergopeptines and semisynthetic ergot alkaloid derivatives, e.g. cabergoline and pergolide (Cvak, 1999). In 2010, the total world production was about 20,000 kg, into which the field cultivation contributed about 50% (Vít Kubesa, Teva Czech Industries, Opava, Czech Republic; personal communication).

The majority of characterized strains of *C. purpurea* and other *Claviceps* species are able to synthesize ergot alkaloids only during the parasitic part of their life. The infection of the plant host by fungal spores initiates the sexual part of the *Claviceps* life cycle. Emerging hyphae invade ovary in the florets and then start to produce masses of new spores and sugar-rich exudates. Approximately 2 weeks later, affected ovaries are transformed into sclerotia where plectenchyma fungal cells initiate the ergot alkaloid biosynthesis (Tudzynski and Scheffer, 2004). Long-term breeding by random mutagenesis and the selection of highly-producing strains of *C. purpurea* allowed an increase in the ergot yield from 400 kg per hectare in the

1940s to over 1 ton per hectare nowadays. The traditional method of ergot production is based on spraying a conidial suspension onto a field-cultivated rye (*Secale cereale*). From the 1990s, hybrid rye lines with induced male-sterility, such as *Hyclaro* (Rentschler Biotechnologie, Laupheim, Germany), were introduced as the host because their unpollinated florets stay open longer and thus extend the period of susceptibility to ergot infection, which leads to a significantly better yield of generated sclerotia (Németh, 1999). Moreover, under natural conditions, *C. purpurea* usually attacks unfertilized ovaries and the process of infection obviously mimics the course of pollination (Tudzynski and Scheffer, 2004). An important benefit of the field ergot production is the wide variability of strains available for the production of specific types of alkaloids and their better genetic stability compared to the mutant strains used for submerged cultures. The yield of field production can reach 1–2 tons of sclerotia and from 10 kg (Tudzynski et al., 2001) to 20 kg (TEVA Czech Industries, in present) of ergot alkaloids per hectare. However, the field production can be dramatically influenced by climatic conditions of the particular year as well as by the quality and uniformity of used hybrid rye. Hence, unfavorable combinations of these factors may even result in a seasonal failure of the production (Vít Kubesa, personal communication).

1.4.3. Production in fermentation plants

Regarding the industrial production of ergot, there are reports on the usage of stationary surface cultivations and submerged cultures, the latter being nowadays a dominant method how to grow the *Claviceps* fungus independently of its host. A stationary cultivation using plastic bags was developed in the 1970s and used for both alkaloid production (Kybal and Vlček, 1976) and preparation of inoculums for field cultivation (Malinka, 1999).

Only some selected mutant strains have the ability to produce alkaloids in submerged cultures; the production strategies were extensively reviewed by Malinka (1999). Cells with this ability, which are called sclerotia-like cells, are shorter and thicker than the normal ones with a robust cell wall and large vacuoles (Spalla, 1973). However, a degeneration process that leads to a loss of the sclerotia-like cell morphology and lowered alkaloid production is relatively frequent; therefore a continuous selection is needed to maintain a good production strain (Malinka,

1999). Fermentation is nowadays used mostly to produce paspalic acid, which isomerizes to D-lysergic acid, and some derivatives of paspalic acid that serve as a starting material for preparation of semisynthetic alkaloid derivatives. D-lysergic acid can be also obtained by alkaline hydrolysis of simple amides, ergometrine and even ergopeptines (Rucman, 1976).

Production of ergopeptines by fermentation is much more complicated and requires specific conditions. Cultivation media induce the formation of sclerotial-cell like morphology by two mechanisms, substrate limitation and favoring oxidative metabolism. Alkaloid production requires a non-inhibitory, slowly metabolized carbon source such as mannitol, sorbitol or sucrose at a high concentration (sucrose 300 g/l). Moreover, a high osmotic pressure (10–20 bar) of the medium is prerequisite for the formation of sclerotia-like cells while inhibiting conidiation (Kobel and Sanglier, 1986). *Claviceps* converts sucrose, which is the main sugar in plant phloem sap and serves as a natural nutrient of the fungus, to fructofuranosyl-oligosaccharides. These oligosaccharides are used as an energy source in the late stage of the fermentation, which is the key period for the production of ergopeptines. Production media usually also contain an organic acid of the tricarboxylic acid cycle as a carbon source, which under low phosphate promotes the high level of oxidative metabolism in the *Claviceps* cells that is necessary for the biosynthesis of secondary metabolites.

Biosynthesis of ergot alkaloids in axenic cultures is positively regulated by tryptophan, which acts as a precursor and inducer, and negatively by phosphate and ammonium, which repress biosynthesis (Sočič and Gaberc-Porekar, 1992). Typically, alkaloid synthesis starts after depletion of phosphate from the medium, whereas an excess of phosphate leads to undesired growth-linked repression of alkaloid production. General reviews about ergot alkaloid production in submerged cultures have been published by many authors including Esser and Düvell (1984); Robbers (1984); Křen et al. (1994); Sočič and Gaberc-Porekar (1992), and Flieger et al. (2004).

1.4.4. Genetic transformation of *Claviceps*

The possibility of genetic transformation of *Claviceps* brought a new tool, not only for obtaining useful knowledge about the genetic principles of ergot alkaloid formation and attractive parasitic lifestyle of the fungus by novel studies of knock-out mutants, but also for having an effect on the quality and/or quantity of ergot alkaloid production for industrial purposes.

Undoubtedly, the most popular method for the genetic transformation of *Claviceps* is protoplast transformation in a medium of high osmotic pressure. A successful method based on protoplast preparation using lytic enzymes was first described in 1989 (Engelenburg et al., 1989) and was later improved (Mey et al., 2002). There are many commercially available enzymes or their mixtures suitable for *C. purpurea* protoplast preparation, such as Lysing enzyme and Driselase (Mey et al., 2002) or β-glucuronidase (Keller et al., 1980). Ca^{2+} ions are universal component of the transformation mixture, while the high osmotic pressure can be adjusted by adding PEG (Engelenburg et al., 1989; Mey et al., 2002). Transformants can then be selected based on acquired antibiotic resistance (Engelenburg et al., 1989; Mey et al., 2002) or by using pyrimidine auxotrophy (Smit and Tudzynski, 1992). Hygromycin (1.0 mg/ml; Comino et al., 1989) and phleomycin or bleomycin (0.1 mg/ml; Mey et al., 2002) are the most often used selection antibiotics. The genes responsible for acquired resistance have been described as *Hph*, encoding hygromycin B phosphotransferase, and *ShBle* gene, coding for a protein binding to bleomycin and inhibiting its DNA cleavage activity (Dumas et al., 1994). Selective growth of transformed vs. untransformed mycelia on a medium containing phleomycin is shown in Fig. 5D (Hulvová et al., 2009).

Technical procedures for *C. purpurea* transformation with the usage of both homologous and non-homologous recombination have been developed (Comino et al., 1989; Smit and Tudzynski, 1992). For the expression of transgenes, constitutive fungal promoters, such as *trpC* (Engelenburg et al., 1989) and *gpdA* from *Aspergillus nidulans* (Lorenz et al., 2010; Scheffer et al., 2005), are used.

Transformation of *C. purpurea* by *Agrobacterium tumefaciens* has been attempted, but no successful procedure has been described yet, although many procedures of *Agrobacterium*-mediated transformation of filamentous fungi

are already known (Groot et al., 1998; Meyer et al., 2003; Michielse et al., 2005; Mullins et al., 2001). Because of the advantages of this method, such as one copy insertion or stable transformant preparation, development of the procedure for *Claviceps* transformation would be very useful.

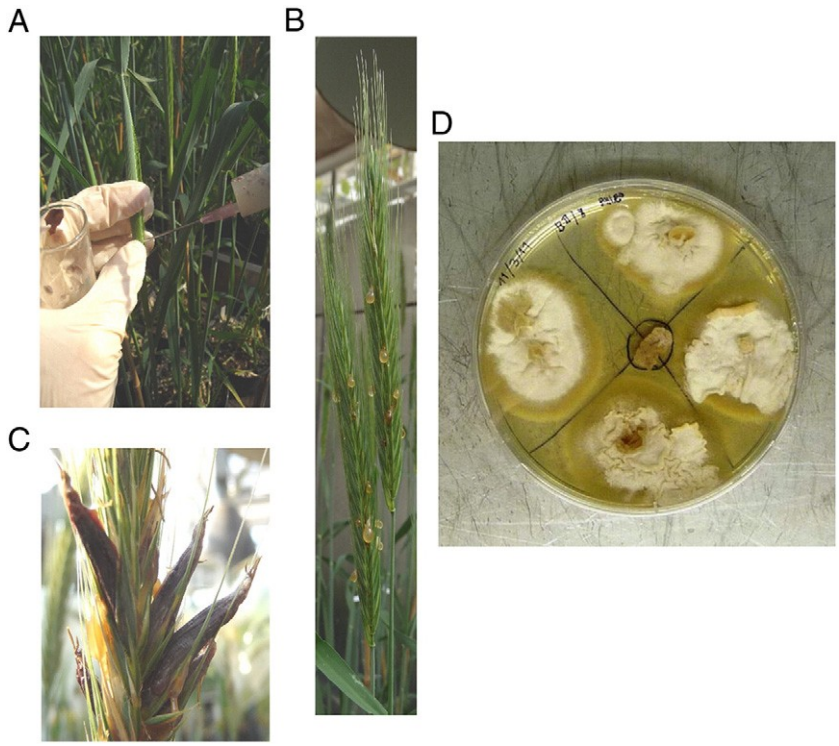


Figure 5. Various stages of the growth of *Claviceps purpurea*: (A) inoculation of rye with conidia suspension, (B) honeydew production on rye, (C) matured sclerotia growing on rye, (D) selection of *Claviceps purpurea* transformants on a Petri dish: protoplasts of the industrial strain Gal 404 were transformed with a linearized plasmid bearing *ble* gene conferring phleomycin resistance; a selective growth of transformed mycelia (on the plate periphery) contrary to untransformed mycelia (in the center) on BII agar plate with 100 µg/ml phleomycin.

1.5 Biotechnological prospectives of production improvement

Improvement of biotechnological processes relies on various transformation approaches used during the last several decades. Up to now, only randomly mutated strains of *C. purpurea* have been exploited in the industrial production of ergot-stock. A sequenced EAS cluster of fourteen genes sheds more light on the metabolic pathways and regulations of ergot alkaloid production. Thus, a directed overexpression of certain genes or targeted up-regulation of the whole cluster could

significantly increase the yield of ergot alkaloids.

Production amounts and a qualitative composition of synthesized alkaloids vary significantly among different isolates of the species *C. purpurea* (Pažoutová et al., 2000). Long terminal repeat sequences and nonautonomous transposons were detected within the ergot alkaloid cluster indicating that spontaneous rearrangements can occur very frequently. Moreover, the cluster for ergot alkaloid production, in *A. fumigatus*, was found to lie in the telomeric region, which is often subject to recombinations, chromosomal breaks and duplications (Coyle and Panaccione, 2005). A recent evolutionary study revealed that the *dmaW* gene passed through many duplications and losses (Liu et al., 2009). The genes of ergot alkaloid cluster are probably also frequently subjected to loss of function mutations, deletions etc. Such an event can be demonstrated on a pseudogene of *dmaW* that was found in an unspecified strain of *C. purpurea* (GenBank AJ312752).

1.5.1. Manipulation of EAS cluster of *Claviceps*

Genes encoding all the enzymes participating in ergot alkaloid biosynthesis are likely to be located within the EAS cluster (Fig. 2C). An evolutionary study of the alkaloid gene cluster has been accomplished including *Claviceps* species that produce different spectra of alkaloids (Lorenz et al., 2007). *A. fumigatus* cluster, whose end-products are clavine alkaloids, contains all ORFs present in the EAS cluster of *C. purpurea* except for the three genes coding for non-ribosomal peptide synthases, *cloA* gene and a functional form of *easH*. Moreover, frameshifts and partial truncations were found in two ORFs for monooxygenases that are responsible for the final production of D-lysergic acid from a chanoclavine precursor. The comparison of *Claviceps hirtella* and *C. fusiformis* is particularly interesting. Despite their very close relationship, the former fungus can synthesize lysergic acid derivatives, while the latter one lacks this ability. The *C. hirtella* cluster encodes an additional functional monomodular non-ribosomal peptide synthase and two monooxygenases, allowing the production of ergometrine, a single amino acid derivative of D-lysergic acid (Lorenz et al., 2009). In general, the overall organization of the cluster is highly conserved; however rearrangements and point mutations dramatically change the expression and functionality of the enzymes involved

in the pathway and consequently both quality and quantity of produced alkaloids. A detailed characterization of alkaloid gene clusters in other members of the genus *Claviceps* is needed as for instance main products of *C. africana* and *C. gigantea* are unique dihydroclavine derived alkaloids. Hence, a combination of different production strains can improve the quality of produced ergot-stock in biotechnological applications.

The gene *dmaW* is common to all *Claviceps* species described to date; its product catalyzes the first specific step in ergot alkaloid biosynthesis and has the key regulatory function in the pathway (Wang et al., 2004). Feeding experiments with tryptophan led to increased activity of DMAT synthase, which resulted in increased total amount of produced alkaloids (Krupinski et al., 1976). Thus, one can expect that ubiquitous overproduction of DMAT synthase enzyme can result in a higher accumulation of dimethylallyltryptophan and reinforcement of further metabolic steps. The actual concentrations of L-tryptophan and dimethylallylpyrophosphate do not seem to be a limiting factor as they are products of the primary metabolism.

The *EasC* gene, with a high homology to catalases, is another gene of interest in the ergot alkaloid cluster. A knock-out mutant of *C. purpurea* did not produce any alkaloids and the transcripts of other cluster genes were down-regulated (Haarmann and Tudzynski, 2006). An elimination of the *easC* gene in *A. fumigatus* led to the same effects and an exogenous addition of chanoclavine to the growth medium restored the alkaloid production. Hence, the *easC* gene product plays an essential role in the metabolic pathway upstream of chanoclavine (Goetz et al., 2011). Hypothetical catalase easC can either consume hydrogen peroxide generated by chanoclavine synthase or function as a general regulation factor of the whole metabolic cascade (Goetz, 2008).

Unlike other secondary metabolic clusters in fungi, no transcription factor gene has been identified among the ergot alkaloid cluster ORFs. Originally, the protein encoded by the *easG* gene was thought to be a good candidate since it shows a homology to Nmr proteins that are involved in regulation of nitrogen assimilation (Tomsett et al., 1981). However, easG was very recently found to catalyze the final step of chanoclavine-I aldehyde conversion to agroclavine (Matuschek et al., 2011).

Based on the published finding, overexpression of either *easC* or *easG* gene was predicted to promote ergot alkaloid biosynthesis in *C. purpurea*. Recently, we used a protoplast method to prepare *C. purpurea* mutants overexpressing genes *easC* and *easG* under a constitutive promoter of glycerol-3-phosphate dehydrogenase

(GAPDH) from *A. nidulans* (Hulvová et al., 2010). Confirmed transformants of each gene were applied on rye growing in a greenhouse, but chemical analysis showed no significant increase in the production of ergot alkaloids. However, the native expression of the two genes in the wild type *C. purpurea* was already very strong (Hanosová et al., unpublished results).

Genetic engineering of the proteins involved in related signaling cascades appears to be another promising way on how to influence ergot alkaloid biosynthesis. Since ergot alkaloid production is dependent on a high osmotic pressure in the culture medium or under natural condition on the production of honeydew, one can expect that receptors and transmitters active in osmoregulation can be good candidates. Pioneering research at this area has been already initiated (Lorenz et al., 2009; Mey et al., 2002).

In fungi, secondary metabolite clusters are often located in a heterochromatin, near the telomere region (Galagan et al., 2005; Nierman et al., 2005; Rehmeyer et al., 2006). In *Aspergillus* spp., expressions of many biosynthetic gene clusters are regulated by histone methyltransferase LaeA. An overexpression of the *laeA* gene in *A. nidulans* led to an increased expression of the cluster genes and product formation (Bok and Keller, 2004). The overexpression of *laeA* gene from *A. nidulans* in the *C. purpurea* P1 strain did not lead to increased expression of EAS cluster genes or ergot alkaloid production (Lorenz et al., 2009).

1.5.2. Improvement of rye hybrid lines with male-sterility

Today, hybrid rye lines with induced cytoplasmic male sterility are widely used in different breeding programs and for heterosis seed production. Fertility can be restored by a suitable parent line bearing restorer gene(s) (Geiger and Miedaner, 2009). Commercial hybrid rye lines with cytoplasmic male-sterility are currently the only suitable host for ergot stock field production. The most crucial factor biasing the quantity of annual ergot yield, besides the weather conditions which influence the progression of the infection in flowering rye plants, is the purity of the rye line. To keep the cytoplasmic male sterility line, regular crossing with the restorer line has to be performed with vigorous selection of male-sterile seed stock. This procedure is very agronomically demanding and significantly elevates production costs. Even

though, maintenance of high-quality seed stock offering 100% male-sterile plants for ergot production is almost impossible because rye is an effective wind pollinator and transfer of pollen containing restorer gametes to long distances can occur on either ergot production field or hybrid seed production field. To avoid losses in ergot production by unexpected leakage of pollen, fields for production of hybrid seed and ergot stock should be located out of traditional rye-growing areas.

Ectopic expression of the barnase gene, ribonuclease from *Bacillus amyloliquefaciens*, in tapetum cells of developing anthers leads to destruction of pollen and therefore to male-sterile plants. A system utilizing the barnase gene and the fertility-restorer gene, barstar, has been introduced to many crops and is one of the most widely used transgenic approaches in modern agriculture (Williams, 1995). Due to the fact that rye is one of the most recalcitrant plant species for tissue culture and genetic transformation (Popelka and Altpeter, 2003), and low economic impact of rye cultivation in global agriculture, transgenic rye lines bearing barnase–barstar system have not yet been prepared. Thus, introduction of the barnase gene into the rye genome together with the linked selection gene (e.g. herbicide resistant) can produce an easy, selective and inexpensive system for obtaining a pure male-sterile rye line suitable for ergot production in the future. Seed stock of the sterile line can be maintained by pollination crossing with wild type plants. The barnase gene, as well as a selective marker, act as dominant alleles in the transgenic genome and thus a 100% sterile population of rye plants can be selected every year by proper application of herbicide.

1.5.3. Susceptibility of rye ovaries to the infection by different *Claviceps* strains, pathogenicity markers

Susceptibility of rye plants to the infection by ergot is another key determinant of a good yield in the field production of ergot alkaloids. The infection depends on many agronomical parameters, but primarily on the fertility of the host cultivar, its pollen shedding and duration of flower opening. For direct testing of the level of pathogen virulence, male-sterile hybrids grown in the greenhouse conditions are used. Several pathogen knock-out transformants were prepared to study the molecular aspects of virulence and host–pathogen interaction. In general,

virulence associated genes can be divided into three main categories, those encoding the enzymes participating in degradation of host cell wall; enzymes scavenging reactive oxygen species that are produced by the host as a pathogen protection response; and various regulatory proteins involved in signaling pathways or functioning as transcription factors. *Claviceps* proteins known to be important for fungal virulence and pathogenicity are listed in Table 1.

As the fungus mimics pollen tube growth during the infection, only mild or no host defense reaction is usually observed (Tudzynski and Scheffer, 2004). On the other hand, high-throughput screening of EST clone libraries from infected rye tissues revealed expression of various proteins related to general pathogen defense responses (Oeser et al., 2009).

Gene	Protein	Function	Reference
Pg1/2	Polygalacturonase	Pectin degradation in rye ovary	Oeser et al. (2002)
Rac	GTPase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Xyl2	Endo-1,4-β-xylanase	Hydrolysis of xylans	Tudzynski and Scheffer (2004)
Cdc42	GTPase	Sensing of polarity and sporulation	Scheffer et al. (2005a)
Cla4	P21-activated kinase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Cot1	Ser/Thr kinase	Sensing of polarity and branching	Scheffer et al. (2005b)
Mid1	Ca^{2+} channel	Signaling Ca^{2+} uptake after membrane Distension	Bormann and Tudzynski (2009)
Mk1/2	MAP kinase	Pathogenicity related signaling	Mey et al. (2002a) Mey et al. (2002b)
Tf1	CREB-like TF	Oxidative stress responses	Nathues et al. (2004)
Hk2	His kinase	Signaling of oxidative stress and osmosensing	Nathues et al. (2007)
Nox1	NADPH-oxidase	ROS generation	Giesbert et al. (2008)

Table 1. Virulence and pathogenicity-related proteins of *Claviceps*.

Part II - Improving field production ofergot alkaloids by application of gametocide on rye host plants

2.1 Abstract

Ergot alkaloids are widely used in the pharmaceutical industry in drug preparations for treating migraines and Parkinson's disease, inducing uterine contraction, and other purposes. Phytopathogenic fungi of the genus *Claviceps* (e.g. *C. purpurea*) comprise a major biological source of ergot alkaloids. Worldwide industrial production of these alkaloids derives almost equally from two biotechnological procedures: submerged culture of the fungus in fermenters and field parasitic production in dormant fungal organs known as sclerotia (also termed ergot).

Ergot yields from field cultivation are greatly affected by weather and also can be much reduced by pollen contamination from imperfectly male sterile rye, as only unfertilized ovaries can be infected by *C. purpurea* spores.

Two substances with gametocidal effect – maleic hydrazide and 2-chloroethylphosphonic acid – were tested during three consecutive seasons in small field experiments for the ability to induce or amplify the male sterility of rye as well as the impacts on germination of *C. purpurea* spores and general vitality of rye host plants. Maleic hydrazide was proven to be a highly effective gametocide on both a fertile rye variety and a variety with imperfectly induced cytoplasmic male sterility. It showed negligible effect on germination of *C. purpurea* spores. Both accurate dosaging of the active gametocidal compound and timing of the application just 2–3 weeks before onset of anthesis proved crucial to achieving high ergot yield with minimum grain impurities.

2.2 Introduction

The biotrophic fungus *Claviceps purpurea* is a member of the globally distributed *Clavicipitaceae* family of plant pathogens and epiphytes. Although the fungus can infect more than 70 grass species, the most susceptible host is rye, *Secale cereal* (Taber, 1985). Infection begins with adhesion of an ascospore transmitted by wind or an insect vector to the stigma of host florets, where it competes with pollen grains. If the spore germinates through the pistil, hyphae start to colonize the ovary and produce conidiospores which spread a

secondary infection to surrounding florets and plants. Flower structures are later transformed into rigid sclerotia, where ergot alkaloids (EAs) begin accumulating approximately 3 weeks after the primary infection (Tenberge, 1999).

Belonging to the class of indole derivatives, EAs are widely used in the pharmaceutical industry for their ability to act as agonists of serotonin, dopamine and adrenergic receptors (de Groot et al., 1998). The genome of *C. purpurea* contains a 68.5 kb cluster of 14 mainly unique genes, the activation of which leads to EA production (Haarmann et al., 2005). EAs can be divided into three major groups: clavine alkaloids; D-lysergic acid and its simple derivatives produced also by other species; and *C. purpurea* exclusive ergopeptines, which are tripeptide derivatives of D-lysergic acid amide. Because organic synthesis of ergopeptines is very difficult and not at all profitable, their production by fungal cells with subsequent extraction and purification is the preferred method of preparation used by pharmaceutical companies. Most *Claviceps* species and strains are able to produce EAs only during their parasitic lives, and that is especially true of those producing ergopeptines as a final product. Industrial strains of *C. purpurea* producing sclerotia containing large amounts of ergopeptines have been obtained through long-term breeding and random mutagenesis. These strains can accumulate as much as 2% of EA in the dry weight of sclerotia. Ergot yields become economically profitable at around one-half ton per hectare of infected rye field (Tudzynski et al., 2001; Ne´meth, 1999) (Teva Czech Industries, recent years; unpublished results).

Parasitic production in the field is based on spraying or injection of non-pollinated rye plants in the earing phase with a conidial suspension of *C. purpurea*. During the 1980s, introduction of hybrid rye lines with cytoplasmic male sterility, such as the cultivar Hyclaro (Rentschler Biotechnologie, Laupheim, Germany) contributed to significantly increased ergot yield (Ne´meth, 1999). These hybrid lines produce no pollen; their florets stay open for a longer period and are thus more susceptible to the infection. Nevertheless, there exist many additional factors which can negatively affect field production. In addition to climatic conditions, the quality and homogeneity of hybrid seed stock are crucial. Even a tiny amount of contamination with the parental component during hybrid seed stock preparation can produce a sufficient amount of pollen

on the production field to cause undesirable formation of grains. Sclerotia formation is then reduced, and that in turn significantly increases the cost of drug preparation. Hybrid sterile lines are maintained by crossing fertile parental components. In as much as one of the lines always carries the genes restoring fertility, individuals of this line must be precisely eliminated during hybrid seed propagation. Cross-contamination with parental components can occur during plant propagation in extreme conditions, where penetration of the genes inducing male sterility is not absolute (Geiger and Miedaner, 2009). Genetic purity failure due to improper agro-technical practices results in the presence of fertile individuals greater than the acceptable threshold of 0.4% in the sterile hybrid population. Such a seed stock with reduced male sterility is then not suitable for commercial ergot production in the field.

For cereals and other crops, male sterility can be also induced chemically. A gametocide is a morphogenetic poison that is typically used to treat the plants in the stage of generative organ initiation (Vorob'ev et al., 2005). It is able to inhibit the creation of microspores in anthers or degrade sporoderm in pollen grains and thus prevent their germination. Depending on dosage, gametocides can cause partial or full male sterility. Determining the appropriate dose is very important, as higher doses can also negatively affect plant growth and the development of female reproductive organs. It is assumed that gametocides induce either a hypersensitive reaction or a phytohormonal imbalance, thereby distorting the development of male gametophyte tissues (Smirnova et al., 1995). Hypersensitive reaction can be induced by substances based upon organophosphates or halogen derivatives of carboxylic acids, while phytohormonal imbalance can be induced by antagonists or agonists of phytohormones, such as 2-chloroethylphosphonic acid, gibberellic acid, and maleic hydrazide. In 1949, Schoene and Hoffman (Schoene and Hoffman, 1949) first described the inhibitory effect of maleic hydrazide (MH; 1,2-dihydro-3,6-pyridazinedione) on plant growth. Later, the compound was successfully used as a male gametocide for many plant species, including coriander (Kalidasu et a., 2009), rice (Lakshmi Praba and Thangaraj, 2005), and wheat (Chopra et al., 1960). MH is an auxin and gibberellic acid agonist (Brian and Hemming, 1957) that is commonly used as a diethanolamine salt or sodium salt soluble in water. MH inhibits the division and extension growth of certain cell types and

prolongs the period of dormancy in many plant species (Haber and White, 1960).

Ethrel (ETH; 2-chloroethylphosphonic acid, also known as ethephon), which upon its being metabolized by the plant is converted into phytohormone ethylene (Domir and Foy, 1978), is the most widely used growth regulator for inducing ripening and enlarging fruit size. Major effects of ethylene include to induce fruit ripening, inhibit extension growth, facilitate the onset of senescence, and, in some species, also to inhibit flowering (Watada, 1986). ETH has been studied for gametocidal effect in many plant species. It is used as a convenient gametocide for wheat, wherein it adequately induces male sterility while maintaining female fertility when applied at an appropriate pre-meiosis growth stage (Sharma and Sharma, 2005). When it was applied to pearl millet, ETH also showed great ability to induce male sterility without negatively affecting female fertility. On the other hand, the susceptibility of ETH-treated pearl millet plants to infection by the parasitic fungus *Claviceps fusiformis* did not change in comparison to that of untreated control plants (Thakur and Rao, 1988).

The aim of the present work was to study the effectiveness of gametocides on the parasitic production of ergot in a series of field experiments. The results obtained show that appropriate application of a gametocide greatly eliminates the ability of pollen to germinate and increases the purity of ergot harvests while maintaining high yields for both a rye hybrid with male sterility as well as classical fertile varieties which previously were assumed to be unsuitable for effective industrial production of EAs.

2.3 Materials and methods

2.3.1. Materials

Winter rye cultivar L25P x L130N with cytoplasmic male sterility was developed by the Plant Breeding and Acclimatization Institute (Radzikow, Poland), and hybrid seeds were propagated from parental components by local farmers. Winter rye population variety Selgo was obtained from Selgen (Stupice, Czech Republic). The *C. purpurea* [Fr.] Tul. strain CCM 8405 mutated by

gamma rays and bred for high EA content is owned by Teva Czech Industries (Valík et al., 2014).

2.3.2. Rye field preparation

The experimental field is located in Olomouc – Holice (Czech Republic; 49834^{0}30^{00} N–17817^{0}07^{00} E). Seeds were sown in the 40th week of the year at a seeding rate of 180 kg per ha into brown-earth soil on small experimental plots, each with an area of 10 m^2. Plants were treated with a herbicide and an insecticide in the same year and again 1 or 2 weeks before the first gametocide application. A mineral fertilizer containing 27% nitrogen was conventionally applied twice during springtime. A field with the fertile rye variety (16 × 2 plots) was approximately 100 m distant from an identically sized field of the sterile variety, both of which were surrounded by border strips of wheat. Plots were treated with Fazor® (Crompton Manufacturing Company; 600 g/kg of MH) or Cerone® (Bayer CropScience; 480 g/L of ETH) at 1-week intervals using a SOLO 485 backpack sprayer in a randomized pattern, each dosage and application time was in two to four replicates. Preparations were dissolved or diluted in tap water and supplemented with the surfactant Rollwet™ (Agrovista, UK) in the final concentration 0.1% (v/v).

2.3.3. Conidiospore preparation and field infection

No sooner than 5 days following the final gametocide application, ears at phenological stage BBCH 63 (for the Selgo variety) and BBCH 61 (for the L25P x L130N variety; Lancashire et al., 1991) were inoculated using conidia suspension of the *C. purpurea* strain CCM 8405. Inoculation was performed by piercing ears with a grid of metal needles regularly dipped into the conidial suspension. The conidial suspension was prepared by homogenizing 4 g of dried mycelia (Valík and Malinka, 1992) using a disperser in 1 L of 0.1% corn starch solution buffered with 0.1 M K^+-phosphate buffer to pH 5.3.

2.3.4. Harvest and yield evaluation

The rye was harvested in the 28th week of the year at phenological stage BBCH 89 using a Hege 160 plot combine. The mixture of grains and sclerotia obtained from each plot was dried separately for 1 month at temperature 30–35°C and then weighed. An amount up to 100 g was manually categorized into grains, small sclerotia (longitudinal size up to 1 cm), and primary sclerotia (larger than 1 cm). Three randomly collected aliquots of 50 g were categorized from a plot upon which yield exceeded 100 g.

2.3.5. Spore inhibition assay

The quantity 0.5 g of mycelium grown on solid sporulation Mantle agar (Mantle and Nisbet, 1976) was added to 250 ml of 150 mM NaCl and homogenized using a disperser at room temperature. The suspension was diluted to concentration 5×10^{10} conidia per ml. Fazor® was added to a final MH concentration corresponding to a series from 2.5 to 10 g/ ml of Mantle agar, upon which it was spread in 0.5 ml aliquots. Plates with identical MH concentrations were grown at 23°C in two replicates. The quantity and morphology of germinating conidia were microscopically evaluated after 24 or 48 hours, respectively.

2.3.6. Ergot alkaloids quantification

Ergot free from any impurities was ground in a ball mill to particle size up to 0.3 mm, and 0.5 g of the product was dissolved in 50 ml of extraction buffer (26% ammonium hydroxide and 90% acetone, 1:100, v/v) on a shaker for 2 hours. A 10 ml aliquot was filtered through glass wool and dried using a vacuum evaporator at 50°C. The residue was dissolved in 2 ml of 90% methanol, filtered through a 0.22 μm nylon filter and analyzed for EA content using ultra high performance liquid chromatography on a Nexera system (Shimadzu) equipped with a C18 reverse-phase column (Zorbax RRHD Eclipse Plus, 1.8 µm, 2.1 mm ID x 50 mm, Agilent). An EA reference mixture was prepared in 90% methanol and contained 0.001% (w/v) of each of the

following alkaloids: ergotamine, ergostine, 8-hydroxyergotamine, ergocornine, *α*-ergokryptine, *β*-ergokryptine, ergocristine, ergogaline, ergotaminine, ergostinine, ergocorninine, *α*-ergokryptinine, *β*-ergokryptinine, ergogalinine, and ergocristinine (all from Teva Czech Industries). The analytes were eluted with solvents A (36 mM triethylamine/phosphate, pH 4.4, and acetonitrile, 4:1, v/v) and B (H_2O and acetonitrile, 1:4, v/v) using the following gradient: 0 min, 9% B; 10–12 min, 9–13% B; 12–14 min, 13–29% B; 14–16 min, 29–44% B; 18–20 min, 44–59% B; 26–28 min, 59–9% B, at flow rate 0.4 ml/min and column temperature of 30°C. Monitoring was at 317 nm.

2.4 Results and discussion

2.4.1. Selection of gametocide with positive effect on ergot yield

Plant protection products commercially available and certified for use in the European Union were surveyed for the presence of active components having gametocidal effect. Finally, two products containing ETH and MH as active compounds, respectively, were selected. These were Cerone®, which is used to increase cereal resistance to lodging, and Fazor®, which is used as a sprouting suppressor for potato tubers or onion bulbs. In the 2012 season, small experimental plots with the hybrid male-sterile rye L25P x L130N were treated with doses ranging from 2 to 15 kg and 2.5 to 160 kg per ha of MH and ETH, respectively, 3 weeks before onset of flowering (BBCH 55, Lancashire et al., 1991). Higher dosages of ETH (above 20 kg per ha) showed such immediate negative effects as formation of leaf lesions, leaf rolling, and cessation of growth and ear development that were visible already a few days after the application. None of these effects were observed on plants treated with MH. Both gametocides caused reductions in plant height across the whole range of dosages used (Table 2).

Dosage (kg/ha) applied at BBCH 51	Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
None	82 ±2	2750 ±308	42.9	57.1	1395 ±106	100.0
Maleic hydrazide						
2	69 ±1	26 ±6	22.9	77.1	484±32	0.9
4	69 ±2	6 ±2	42.6	57.4	193±21	0.2
8	69 ±1	3 ±0	44.4	55.6	127±100	0.1
15	59 ±1	None	None	None	None	0.0
Ethrel						
2.5	64 ±2	1730 ±252	80.7	19.3	674±48	62.9
5	60 ±1	1994 ±704	75.5	24.5	824±93	72.5
10	61 ±1	102±16	66.2	33.8	302±12	3.7
20	55 ±1	86±22	38.8	61.2	294±32	3.1
40	57 ±1	202±41	54.5	45.5	132±29	7.3
80	52 ±1	130±40	50.9	49.1	110±7	4.7
160	51 ±1	74±30	100.0	None	16 ±4	2.7

[a] Measured at phenological stage BBCH 57.

Table 2. Effects of different dosages of MH and ETH on grain production and ergot yield on hybrid rye L25P x L130N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2012 season).

After treated plants as well as non-treated controls were infected by *C. purpurea* conidia, the progress of infection was followed and ergot yield estimated. The first symptoms of infection – formation of honeydew droplets and polyamine odor – were obvious 12 days after infection with the same intensity on control plots as on plots with gametocide dosages up to 4 kg and 20 kg per ha for MH and ETH, respectively. Plots with higher dosages showed significantly lower infection symptoms. The seed stock used (hybrid rye L25P x L130N) showed a high penetration of fertile individuals ranging from 2% to 20%. MH significantly decreased grain yield for treated plants already at a dosage of 2 kg per ha in contrast to non-treated plants (Table 2). ETH treatment caused a similar reduction, but only at much higher doses and while also negatively affecting plant height and vitality already at a dosage of 2.5 kg per ha. Although both gametocides also slightly reduced ergot yield even at the lowest application dosages, the decrease in contaminating rye grains in the total harvest was far more prominent, and especially so in the case of MH. In contrast to ETH, MH did not substantially affect plant vitality or growth. MH was therefore selected for further testing in subsequent seasons. ETH treatment was repeated in the 2014 season with similar results. There was a proportional reduction in plant height and grain and ergot yield with increasing ETH dosage from 1 to 5 kg per ha (data not shown), and the total ergot yield and

grain/ergot ratio were much better when MH was used.

2.4.2 Ergot yield from fertile rye variety treated with maleic hydrazide

Although male-fertile rye varieties have not been used for industrial production in recent decades, some of them have high susceptibility to *C. purpurea* infection, and especially those with a broader range of flowering time and longer ears. It is crucial to infect fertile varieties before pollination onset as the pollen competes strongly with ergot conidia (Ne´meth, 1999). Since rye is cross-pollinating and the onset of flowering in the field is not synchronized, a huge amount of pollen transmitted by wind from early maturing individuals can block *C. purpurea* infection of those ovaries that were not infected early enough.

The fertile rye variety Selgo was treated with MH at different time points, beginning 3 weeks before the onset of flowering. Similarly to the case of the male-sterile variety, MH application visibly affected only plant height while not altering flower development and pollen shedding from anthers. The fertile rye was not inoculated until phenological stage BBCH 62, at which time approximately 30% of the anthers in the field were mature and produced pollen. Despite pollen production, decrease in grain formation by more than 10-fold was observed on all plots where the gametocide was applied (Tables 3 and 4). The MH concentrations used and the application during the period when male gametophytes are already in development most probably did not affect microspore formation. Rather, these led to the formation of pollen grains that were unable to germinate.

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Hybrid rye L25P x L130N with imperfect male sterility					
19th BBCH 47	20th BBCH 49	21th BBCH 55	22th BBCH 59						
–	–	–	–	119 ± 6	1938 ± 370	63.7	36.3	178 ± 22	100.0
–	–	0.5	0.5	112 ± 2	844 ± 71	39.3	60.7	164 ± 114	43.6
–	0.5	–	–	113 ± 2	2245 ± 254	67.0	33.0	197 ± 18	115.8
1	1	1	1	77 ± 1	17 ± 12	66.3	33.7	74 ± 7	0.9
–	1	1	1	116 ± 1	487 ± 354	60.4	39.6	624 ± 72	25.1
–	1	1	–	113 ± 2	1317 ± 175	89.5	10.5	295 ± 19	68.0
–	1	–	–	105 ± 2	1168 ± 369	85.7	14.3	91 ± 9	60.3
–	–	1.5	1.5	108 ± 6	137 ± 48	61.5	38.5	670 ± 125	7.1
–	1.5	–	–	110 ± 2	1078 ± 87	93.5	6.5	217 ± 14	55.6
–	2	2	2	106 ± 1	90 ± 57	78.9	21.1	462 ± 49	4.6
–	2	–	–	108 ± 3	371 ± 154	83.9	16.1	193 ± 15	19.1
–	3	–	–	103 ± 3	197 ± 35	59.4	40.6	271 ± 32	10.2
MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Fertile rye Selgo					
19th BBCH 49	20th BBCH 51	21th BBCH 59	22th BBCH 61						
–	–	–	–	124 ± 3	4301 ± 136	61.1	38.9	190 ± 11	100.0
1	–	1	1	97 ± 3	7 ± 2	35.7	64.3	14 ± 2	0.2
–	1	1	1	118 ± 3	416 ± 113	24.7	75.3	308 ± 38	9.7
–	2	2	2	105 ± 4	114 ± 53	42.1	57.9	428 ± 58	2.7
–	3	3	3	106 ± 1	209 ± 112	27.6	72.4	485 ± 392	4.9

[a] Measured at phenological stage BBCH 69.

Table 3. Effects of different MH dosages and application times on grain production and ergot yield for hybrid rye L25P x L130N with imperfect male sterility and fertile rye variety Selgo infected by *C. purpurea* CCM 8405 (experiment run during the 2013 season).

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Fertile rye Selgo					
17th BBCH 49	18th BBCH 51	19th BBCH 59	20th BBCH 61						
–	–	–	–	127 ± 6	3946 ± 957	58.6	41.4	162 ± 109	100.0
2	–	2	–	110 ± 1	38 ± 29	38.5	61.5	85 ± 61	1.0
2	2	2	–	106 ± 8	101 ± 22	54.2	45.8	69 ± 8	2.6
2	2	2	2	111 ± 1	107 ± 6	59.2	40.8	91 ± 35	2.7
–	2	–	2	122 ± 3	82 ± 40	51.4	48.6	315 ± 67	2.1
–	2	–	–	119 ± 3	493 ± 85	54.9	45.1	190 ± 42	12.5
–	2	2	–	122 ± 3	488 ± 154	50.2	49.8	333 ± 94	12.4
–	–	2	2	127 ± 7	450 ± 138	56.4	43.6	211 ± 6	11.4
3	3	1	1	109 ± 1	45 ± 48	53.8	46.2	34 ± 8	1.1
–	3	1	–	127 ± 2	288 ± 194	60.0	40.0	131 ± 78	7.3
–	4	–	–	123 ± 2	595 ± 6	53.5	46.5	170 ± 27	15.1
–	–	4	4	125 ± 8	171 ± 157	56.4	43.6	170 ± 72	4.3

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Hybrid rye L25P x L130N with imperfect male sterility					
17th BBCH 45	18th BBCH 47	19th BBCH 55	20th BBCH 59						
–	–	–	–	115 ± 4	2822 ± 410	74.7	25.3	24 ± 13	100.0
2	–	2	–	95 ± 5	34 ± 27	54.4	45.6	14 ± 10	1.2
2	2	2	2	89 ± 4	51 ± 8	29.2	70.8	8 ± 2	1.8
2	2	2	–	90 ± 5	19 ± 14	36.0	64.0	5 ± 3	0.7
–	2	–	2	105 ± 2	160 ± 14	53.6	46.4	64 ± 38	5.7
–	2	–	–	107 ± 3	187 ± 6	57.1	42.9	83 ± 16	6.6
–	2	2	–	105 ± 2	202 ± 59	49.9	50.1	102 ± 5	7.2
–	–	2	2	112 ± 2	642 ± 152	61.2	38.8	53 ± 2	22.7

[a] Measured at phenological stage BBCH 69.

Table 4. Effect of different MH application times on grain production and ergot yield for fertile rye variety Selgo and hybrid rye L25P x L130 N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2014 season).

The gametocide also did not affect the development of female gametophytes, as the ovaries remained susceptible to conidiospore infection and produced fully developed sclerotia (Fig. 6A). The ratio between harvested ergot and rye grain changed significantly (Fig. 6B), and ergot yield almost reached the value obtained using the hybrid male-sterile variety (Table 3).

Figure 6. Effect of MH on the infection progress and ergot yield in the experiment from the 2013 season. (A) *C. purpurea* infection on the fertile rye variety Selgo treated with three dosages of MH (2 kg per ha, left) and the non-treated plot (right). (B) Ergot harvest from the plot with the fertile rye variety Selgo treated with MH (3 x 2 kg per ha, left) in contrast to the harvest from non-treated plants (right). (C) Effect of four subsequent applications of 1 kg of MH per ha on the rye hybrid L25P x L130N (left) in comparison with the non-treated plot (right).

Previous studies have shown that sclerotia formed on a fertile rye are generally larger but fewer in number per ear (primary sclerotia) than are those on malesterile rye (mostly sclerotia from secondary infection), the former condition being considered a disadvantage for ergot harvest (Ne´meth, 1999). A different proportion of primary as opposed to that of secondary sclerotia on fertile versus sterile rye treated with MH was also observed in the 2013 season experiment (Table 3). This difference was not so obvious in the following year, however, thus suggesting that the size of sclerotia might be influenced also by weather conditions.

A lower dosage of MH (1–4 kg per ha), which already significantly decreased grain formation for the fertile rye variety, showed almost no effect on ergot yield. On the other hand, the time of first application seemed to be crucial for obtaining a good ergot yield with minimal grain contamination. Therefore, the field experiment in the 2014 season was directed mainly to finding the proper application time.

2.4.3. Application time of maleic hydrazide to maintain high yield and low grain/ergot ratio

Trials with varied times of gametocide application demonstrated the great importance of proper timing. Even the lowest dosage of MH (1 kg per ha) caused a dramatic reduction in the ergot yield on both those varieties used when it was applied earlier than at the beginning of heading, the stage when awns became visible (Table 3). Earlier application caused more distinctive changes than did later application of even three times the lowest dosage. Strong reduction in plant height, darker leaf color, and a delay in inflorescence emerging from flag leaf sheath (Fig. 6C) were observed in plants treated at a phenological stage between BBCH 47 and 49, thus indicating that processes other than pollen germination (e.g. female gametophyte development) might also be affected. The negative effect of earlier application was observed in two consecutive seasons. The positive effect of more than one application is also obvious from both seasons' experiments. Generally, the application of 2 kg per ha in 3 subsequent weeks before the field is in full flowering, but after the ear emerges from the leaf sheath, appears to be the most promising.

Lower ergot yield from the sterile variety in the 2014 season can be attributed to weather extremities. The spring of 2013 was extremely cold and wet with temperatures 0.8°C below and precipitation 116% above 30-year norms (1961–1990). On the other hand, the spring of 2014 had temperatures 2.4°C above and precipitation 86% below norms (Czech Hydrometeorological Institute; http://www.chmi.cz). In 2014, therefore, the development of rye plants was accelerated by approximately 2 weeks. As this estimation might not have been precise, the application of the first and second doses in 2014 was performed a couple of days earlier in relation to the plants' phenological stage. Moreover, fungal infections typically progress much better under cold and wet climate conditions (Ne´meth, 1999), which occurred during the spring of 2013 (in which year higher yields were obtained).

2.4.4. Inhibitory effect of maleic hydrazide on the germination of *C. purpurea* conidia

The inhibitory effect of MH applied in the form of the commercial preparation Fazor® on *C. purpurea* spore germination was tested *in vitro* (Table 5).

MH dosage (kg/ha)[a]	Germinated spores (%)	
	After 24 hours	After 48 hours
0	54	62
1	36	37
2	31	33
2.5	32	35
3	29	31
3.5	15	23
4	11	19

[a] At a concentration in the agar corresponding to an application of 400 L per ha.

Table 5. *In vitro* testing of MH inhibitory effect on *C. purpurea* CCM 8405 conidia germination

The results correspond well to the data obtained from the field experiments. Dosages of 1–3 kg of MH per ha showed only a weak negative effect on the ability of *C. purpurea* conidia to germinate. However, hyphae germinated from spores at MH concentrations above 3 kg per ha were significantly shortened. Nevertheless, if the time lag between infection and final gametocide

application is not less than 5 days, the negative effect of the gametocide on spore germination can be ignored at a dosage around 2 kg per ha.

2.4.5. Ergot alkaloid content in sclerotia produced on rye treated with gametocides

Sclerotia grown on rye treated with MH and ETH during the 2014 experiment exhibited no significant alterations in EA content except for those that originated from plots with the earliest gametocide application (Table 6).

Gametocide dosage (kg/ha) Week of the year				Ergotamine[a] (% of dry mass)	% of control	Total EA (% of dry mass)	% of control
17th	18th	19th	20th	Sclerotia collected from the male-fertile rye Selgo			
–	–	–	–	1.64 ± 0.28	100	2.09 ± 0.23	100
MH application							
–	2	–	2	1.71 ± 0.21	104	2.21 ± 0.27	106
3	3	1	1	1.11 ± 0.10	68	1.45 ± 0.10	69
–	3	1	–	1.52 ± 0.17	93	1.95 ± 0.21	93
–	–	4	4	1.44 ± 0.19	88	1.90 ± 0.15	91
ETH application							
3	–	3	–	1.69 ± 0.39	103	2.14 ± 0.40	102
1	–	1	–	1.50 ± 0.08	91	1.94 ± 0.11	93

Gametocide dosage (kg/ha) Week of the year				Ergotamine[a] (% of dry mass)	% of control	Total EA (% of dry mass)	% of control
17th	18th	19th	20th	Sclerotia collected from the hybrid rye L25P x L130N			
–	–	–	–	1.42 ± 0.22	100	1.82 ± 0.23	100
MH application							
2	2	2	2	0.90 ± 0.21	63	1.16 ± 0.30	64
–	2	–	2	1.33 ± 0.22	94	1.76 ± 0.26	97

[a] Sum of ergotamine and its oxidative product ergotaminine.

Table 6. Effect of selected gametocides on EA accumulation in sclerotia of *C. purpurea* CCM 8405. Ergotamine, ergotaminine, ergostine, ergostinine, 8-OH ergotamine, ergotoxine and ergotoxinine were quantified by ultra high performance liquid chromatography in the extract from a mixture of primary and small sclerotia. Presented are means ± SE of two biological replicates.

Lower EA content in sclerotia obtained from plants where MH was applied at swollen flag leaf sheath stage was most probably due to strongly reduced growth of the plants and limited capacity of their sink. *C. purpurea* strain CCM 8405 is the main producer of the highly valued ergopeptine ergotamine, so it is noteworthy that the gametocides used in this work did not increase impurity

content and ergotamine comprised 75–78% of total EA content. That content was similar to that obtained from sclerotia from non-treated plants. No significant differences in EA composition and quantity were observed between primary and secondary sclerotia (data not shown).

2.5 Conclusion

Streamlining the ergot field production process can lead to a significant reduction in costs associated with manufacturing EA-based drugs. Male sterility that is chemically induced via an appropriate gametocide might minimize the impact of shortcomings in agro-technical practice. The procedure proposed in this work decreases the contents of organic contaminants coming from rye grain in the harvested sclerotia mass, but most importantly it permits the producer to use common fertile rye cultivars. The method of maintaining a population of fully sterile rye and preparing hybrid seed stock is challenging, and every failure by the human operator is followed by a large increase in undesirable grains contaminating the ergot. That means the costs of high-quality male-sterile hybrid seeds are as much as 20 times greater than are those of standard fertile rye cultivars. Plant protection products containing MH, an auxin antagonist with gametocide effect that was found to be the most promising, are readily available and relatively inexpensive. Thus, their application needs not significantly increase the costs of EA field production. Because *C. purpurea* is a living organism, sclerotia yield always varies from season to season, depending mainly on climatic conditions, as is also evident from the differing results obtained in the three subsequent seasons presented herein. Nevertheless, employing the gametocide can significantly increase the purity of the ergot while having little or no impact on its total yield if the application is accurately timed. Teva Czech Industries is presently one of the world's largest producers of EA-based drugs. The company's production is centered mainly on parasitic field cultivation and has generated undesirable losses over the past decade due to the heterogeneity of hybrid rye host plants. The first attempt at large-scale ergot cultivation on a fertile rye variety treated with gametocide brought a promising improvement in the cost-effectiveness of EA production for the 2014 season. Preliminary estimates of ergot yield range between 0.5 and 1 ton per

hectare with organic impurities up to 30% for production at two independent locations (2 and 3 ha areas) using a fertile rye variety treated with two dosages of MH.

Part III –Introduction of an optimized method for the transformation of industrial strains *of Claviceps purpurea*

3.1 Abstract

An optimized method for the transformation of high-producing *C. purpurea* industrial strains was introduced in order to produce a large number of transformants per transformation. The method was used for the generation of two independent Gal 404 *LpsA2* knockout mutants and several overexpressors of ergot alkaloid synthesis (EAS) cluster genes.

In order to define the effect on the quantity of the alkaloids produced, three selected genes of the EAS cluster were expressed constitutively under the control of a strong gpdA promoter in the Gal 404 strain and/or in Gal 130 strain, respectively. The verified transformants were inoculated on sterile rye plants and the sclerotia were harvested at two time points during infection, followed by alkaloid content analysis. HPLC analysis did not show any statistically important changes in the ergot alkaloid production of the transformants. The *LpsA1* gene from the ergotamine-producing 20.1 reference strain was constitutively expressed under the control of a PoliC promoter in Gal 130, an ergocristine-producing strain, resulting in significant changes in the relative representation of ergotamine in the EA pool in two independent transformants.

3.2 Introduction

A method of transformation producing a large number of transformants is essential “know-how” during the homologous recombination-based preparation of knockout mutants, because in *Claviceps purpurea*, homologous recombination occurs in 1-2% of the transformed cells (Haarmann et al., 2008). The method of transformation from Mey et al., 2002, optimized in Hulvova, 2009, provides 1-2 transformants per transformation if the Gal 404 strain is transformed. The introduction of a method optimized in order to produce a large number of transformants per transformation was a necessary step for the continuation of reverse genetic research focused on *C. purpurea*, industrial strains.

Ergot alkaloids are L-tryptophan-derived secondary metabolites produced by fungi of the phylum *Ascomycota* (Wallwey and Li, 2011), including members of the *Clavicipitaceae* family as a representative of EA producers. They can be divided into three groups, i.e. clavins, D-lysergic acid amides and ergopeptides.

The first step in the EA biosynthetic pathway is the conversion of L-tryptophan and dimethylallyl pyrophosphate to dimethylallyl tryptophan by enzyme dimethylallyl synthase (Gebler and Poulter, 1992) encoded by the *dmaW* gene (Tudzynski et al., 1999). The action of the products of the *easF, easC* and *easE* genes leads to the biosynthesis of chanoclavine-I (Rigbers and Li, 2008; Lorenz et al., 2010), which is subsequently converted to chanoclavine-I aldehyde by the product of *easD* (Wallwey and Li, 2011). The cyclization of chanoclavine-I aldehyde by the *easA* gene product (Coyle et al., 2010) and *easG* gene product (Matushek et al., 2011) leads to the biosynthesis of agroclavine. Multiple reactions leading to the biosynthesis of D-lysergic acid from agroclavine are catalyzed by the product of the *cloA* gene (Haarmann et al., 2006; Robinson and Panaccione, 2014). Nonribosomal peptidyl synthetase (NRPS) LPSB activated D-lysergic acid is transferred to NRPS LPSA, followed by the formation of tripeptide thioester intermediate and the release of D-lysergyl tripeptide lactam (Riederer et al., 1996; Walzel et al., 1997), which is converted to ergopeptide by the *easH1* gene product (Havemann et al., 2014).

The genes of the biosynthetic pathway of ergot alkaloids are localized in a cluster discovered by Tudzynski et al. in the P1 strain of *C. purpurea* by chromosome walking (Tudzynski et al., 1999). Homologs of some of the cluster genes are present in other species. According to their presence and functionality, species differ in terms of the alkaloids produced; e.g. nine homologs in *Claviceps fusiformis* with the lack of a functional copy of NRPS genes result in the production of clavine alkaloids as the final metabolites of the pathway (Lorenz et al., 2007).

Ergot alkaloids are natural substances, widely used in the pharmaceutical industry because of their biological activity, resulting from their affinity to receptors for the neurotransmitters serotonin, dopamine and adrenaline (reviewed in Sharma et al., 2016). Because of their pharmaceutical usage, fungi-producing ergot alkaloids became a target group for improvement by genetic engineering methods. For a long time, random mutation and breeding were the main methods used for the improvement of industrial strains. Strains producing high levels of alkaloids, as well as strains producing alkaloids during submerged cultivation under specific conditions, were prepared in this way.

The discovery of the cluster of genes responsible for the production of ergot alkaloids, describing the function of all the genes that are present (reviewed in

Gergards et al., 2014; Young et al., 2015) and genome sequencing of the main producers of ergot alkaloids (Schardl et al., 2013) opens up the possibility of targeted genetic manipulation.

Overexpression of the gene-encoding enzyme for the limiting step of the pathway determining the rate of secondary metabolite production can lead to the acceleration of secondary metabolite production. Potential limiting steps could be catalysed by enzymes encoded by *dmaW*, *easC* and *easG* genes. The *dmaW* gene, encodes enzyme catalyzing the first step of the biosynthetic pathway of ergot alkaloids, dimethylallyltryptophan synthase (Tudzynski et al., 1999). The *easC* gene encodes catalase, which is involved in chanoclavine-I synthesis (Goetz et al., 2011). It was one of the two most up-regulated genes of the cluster, when the transcriptomes of alkaloid-producing structures – sclerotia - and low-productive structures – mycelia – were compared (Majeska et al., 2016). *EasG* gene encodes enzyme catalyzing the conversion of chanoclavine-I aldehyde to agroclavine (Matuschek et al., 2011).

The *LpsA* and *LpsB* genes encode non-ribosomal peptide synthases (NRPS), responsible for the connection of D-lysergic acid with three amino acids, therefore ergopeptide biosynthesis. D-lysergic acid attached to LPSB is linked to bicyclic tripeptide, attached to LPSA in an amide-like fashion (Correia et al., 2003) or to LPSC-activated alanine (Ortel and Keller, 2009).

The *Claviceps purpurea* EA cluster consists of four NRPS genes: *LpsB, LpsC*, the ergonovine synthetase, responsible for alkylamide ergonovine production (Ortel and Keller, 2009), and two *LpsA* genes, encoding products, that are catalyzing the biosynthesis of ergopeptides (Haarmann et al., 2005). The type of the first two amino acids present in tripeptide moiety determines the type of the final ergopeptide. The third amino acid is nearly always proline.

It was described in Riederer et al., 1996, by feeding studies of an isolated LPSA enzyme from the D1 strain of *C. purpurea*, that the substrate specificity of the enzyme is not absolute. Keller indicates that it is mainly the module responsible for position I that has broad substrate specificity and, like Riederer, mentions the dependence of the type of the final ergopeptide on the actual concentrations of the relevant amino acids in the cellular pool (Keller, 1999). Nevertheless, on the evidence of knockout studies of *LpsA1* in the P1 strain, a new hypothesis was created, according to which absolute substrate specificity is expected, meaning that

each LPSA enzyme catalyzes the formation of just one type of ergopeptide (Haarmann et al., 2008). On the basis of this study, a number of recent publications (Lorenz et al., 2009; Gerhards et al., 2014) suggest the biotechnological improvement of industrially used strains by the knockout of one *LpsA* gene for single alkaloid production.

Very recently, the adaptation of a theory partially based on original hypotheses has been discussed. Transcriptome sequencing of the four industrial EA producers with different spectra of alkaloids produced showed significant differences in the expression of the genes involved in the biosynthesis of several amino acids, varying across strains with different spectra of alkaloids produced (Majeská et al., 2016). Many well-described strains produce much higher numbers of types of ergopeptides than the number of *LpsA* genes that are present (there are usually two *LpsA* genes present in the strains described so far), even if they are produced in small amounts (see the description of the Gal 404 and Gal 130 strains in “Material and methods”). Therefore, it is appropriate to modify the hypothesis and consider the expansion of the substrate specificity of the enzyme.

Heterologous expression of the *LpsA* gene (if the donor strain differs from the acceptor strain in the EA spectra produced) can contribute to the clarification of the substrate specificity of the enzyme.

3.3 Material and methods

The description of the transformation method is related to the Gal 404 strain, but identical procedure was applied to the Gal 130 strain.

3.3.1. Description of strains of microorganisms used in this study

C. purpurea, Gal 404 (patent CZ287130) and *C. purpurea*, Gal 130 (patent CZ279877) prepared by random mutagenesis and maintained by breeding were kindly provided by Teva Czech industries, s.r.o., Opava-Komárov, Czech Republic for research purposes. The Gal 130 strain is one producing industrially high amounts of ergocristine. Its alkaloid spectra consist mainly of ergocristine, containing minor produced alkaloids: α ergokryptine, β ergokryptine, ergotamine, ergogaline, ergocornine and their stereoisomers –inins. Gal 404 is a

strong industrial ergotamine producer. Its alkaloid spectra consist mainly of ergotamine, containing minor produced alkaloids: ergotoxine, ergocristine, ergogaline, ergostin and their stereoisomers –inins.

The 20.1 strain (described in Schardl et al., 2013), used for the amplification of the *LpsA1* gene, the ergotamine and ergocryptine producer, was kindly provided by Prof. Tudzynski for research purposes.

Escherichia coli, TOP10 was purchased from Invitrogen, USA.

The FGSC 9721 strain of *Saccharomyces cereviceae* described in Winston et al., 1995 was kindly provided by Prof. Tudzynski.

3.3.2. Culture cultivation conditions

50 ml of liquid BII media (Esser et al., 1978) in a 250-ml Erlenmeyer flask was inoculated with Gal 404 strain culture (or the Gal 130 strain), growing on solid BII media. The liquid culture was cultivated for three days at 26 ˚C and 180 RPM.

Mantle agar (Mantle et al., 1976) was used as solid media inducing the sporulation of the Gal404 and Gal130 strains, needed for monosporic isolation and plant inoculation. The culture was grown for two weeks before the harvesting of the spores.

The yeast recombinational cloning was performed with the FGSC 9721 yeast strain (Winston et al., 1995) incubated at 30 °C in yeast extract-peptone-dextrose (YPD).

Standard cloning was performed with TOP10 strain of *E. coli bacteria*, incubated at 37 °C in LB media.

3.3.3. Vector description

pDrive plasmid (QIAGEN, Hilden, Germany).

GPDA_PROMO_pMK was commercially synthesized by Mr.GENE (http://mrgene.com).

The P434 and P444 vectors (Fungal Genetics Stock Center, http://www.fgsc.net/) were used for the preparation of hygromycin-resistant and phleomycin-resistant transformants by an optimized method for transformation. The P444 vector was used for the preparation of the vectors for the strong constitutive expression of the *dmaW*, *easC* and *easG* genes.

pRS426 (Colot et al., 2006) was used for the yeast recombinant cloning of the Gal404:Δ*LpsA2* vector.

The pNDH-OGG::lpsA1 gfp- vector was provided by Mgr. Michaela Hradilová; the preparation is described in Hradilová, 2016.

3.3.4. Molecular cloning

The primers were synthesized and delivered by Sigma-Aldrich, St. Louis, Missouri, USA). All the PCR reactions for the molecular cloning purposes were performed with proofreading, Phusion polymerase (NEW ENGLAND Biolabs, Ipswich, Massachusetts, USA). The PCR products were purified using the GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich). The primers used for cloning the vectors are listed in Table 7.

The Gal 404:Δ*LpsA2* vector was constructed using the yeast recombinational cloning method. The regions covering the areas of the left flank and right flank were amplified using the 5F_lpsA2_GAL404, 5R_lpsA2_GAL404 and 3F_lpsA2_GAL404, 3R_lpsA2_GAL404 primers. The primers contain overlapping sequences towards the yeast shuttle-vector pRS426. The PCR products, the linearized pRS426 and phleomycin-resistant cassette (amplified with the CpBleF1 and CpBleR1 primers from pRS426) were transformed into the FY834 yeast strain for homologous recombination.

The *easC* gene was amplified from the genomic DNA with the easCfull_fw and easCfull_rev primers, and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, and digested with

EcoRI and ligated with EcoRI digested GPDA_PROMO_pMK (Hulvova, 2009), providing *easC*-GPDA_PROMO_pMK plasmid. The *easC*-GPDA_PROMO_pMK with *easC* integrated in positive direction was XbaI and Sac II digested and ligated with XbaI and Sac II digested p444, giving rise to a p444-*easC* vector (Fig. 7A), where the *easC* gene is localized between the gpdA promoter (the promoter for glyceraldehyde-3-phosphate dehydrogenace from *Aspergillus nidulans*) and the TrpC terminator from the same organism.The *easG* gene was amplified from the genomic DNA with the EasG_full_fw and EasG_full_rev primers and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, followed by XhoI, KpnI digestion and ligation with XhoI and KpnI digested GPDA_PROMO_pMK, providing *easG*-GPDA_PROMO_pMK. The e*asG*-GPDA_PROMO_pMK was SacII and XbaI digested and ligated with SacII and XbaI digested p444 plasmid (analogously to p444-*easC*), giving rise to a p444-*easG* vector (Fig. 7C).

The *dmaW* gene was amplified with the DMaWfull_fw and DMaWfull_rev primers and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, followed by EcoRI digestion and ligation with EcoRI digested GPDA_PROMO_pMK, providing *dmaW*-GPDA_PROMO_pMK. The *dmaW*-GPDA_PROMO_pMK with *dmaW* integrated in positive direction was SacII and XbaI digested and ligated with SacII and XbaI digested p444 plasmid (analogously to p444-*easC* and p444-*easG*), giving rise to a p444-*dmaW* vector (Fig. 7B).

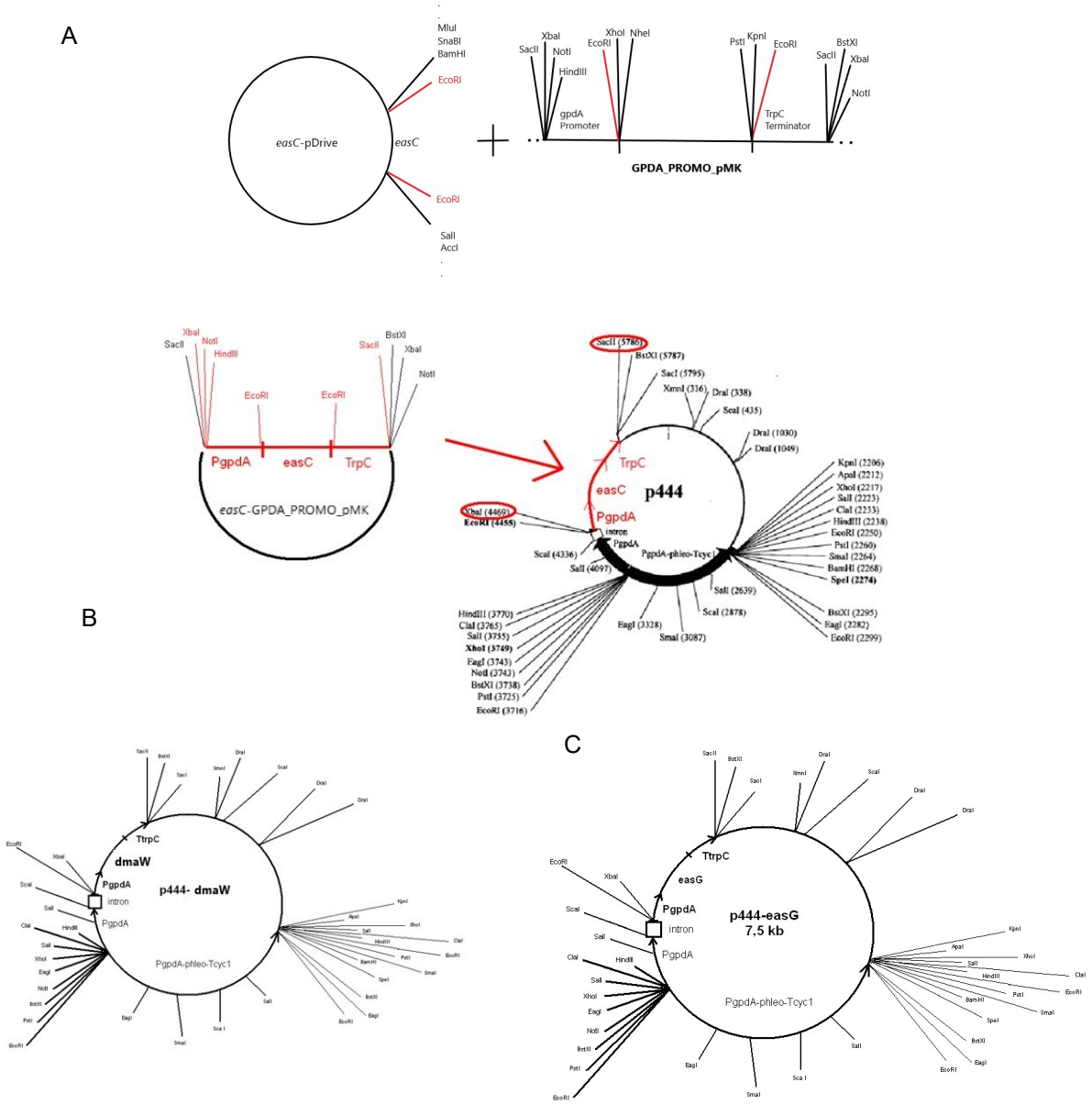


Figure 7. (A) Molecular cloning of the p444-*easC* vector, (B) vector map of p444-*dmaW,* (C) vector map of p444-*easG*.

3.3.5. Transformation of *Claviceps purpurea*

The liquid culture in BII medium was centrifuged for 10 minutes at 5600g, RT and washed twice with SMaC buffer (0.85 M sorbitol, 0.2 M potassium maleate, 50 mM $CaCl_2$ pH 5.2, Mey et al., 2002).

The protoplast-generating solution was prepared by dissolving 5 mg/ml Lysing enzyme (*Trichoderma harzianum*, Sigma-Aldrich) in SMaC buffer, followed by sterilization by filter membranes with a pore size of 5 µm (Whatman® PTFE membrane filters, Sigma-Aldrich) and 0.22 µm (Millex syringe-driven filter unit, Sigma-Aldrich). The cell precipitate was re-suspended in 20 ml of protoplast-generating solution and incubated for two hours at 28 ˚C, with shaking at 80 RPM. Incubation was followed by filtration with a Nytex membrane (Franz Eckert, GmbH, Waldkirch, Germany), getting rid of the cell debris. The protoplast suspension was centrifuged at 900 g, RT for 10 minutes and washed twice with STC buffer (0.85 M sorbitol, 10 mM Tris, 50 mM $CaCl_2$, pH 7.5, Mey et al., 2002). The protoplast pellet was dissolved in 300 µl of STC buffer. The quality, purity and concentration of the protoplasts were determined under the microscope in a Bürker chamber.

The protoplasts obtained were transformed as follows:

10 µl of linearized DNA with a concentration of 1 µg/µl were added to 90 µl of STC buffer, followed by the addition of 50 µl of PEG solution (25% PEG 6000, 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.5, Mey et al., 2002) sterilized by filtration through a 5 µm-pore size filter membrane and 100 µl of protoplast solution. After 20 minutes of RT incubation, another 2 ml of PEG solution was added to the mixture and the transformation was finished after incubation lasting five minutes by the addition of 4 ml of STC.

625 µl of the cell solution after the transformation procedure was mixed with 20 ml of BII agar for regeneration (pH 8, no Fe^{2+} added, 200g/l sucrose).

One petri dish was antibiotic free, serving as a control of the vitality of the cells and ability to regenerate. In the case of the preparation of phleomycin-resistant transformants (transformation performed with linearized p444 vector), the rest of the petri dishes were prepared by mixing cells with BII agar for regeneration containing 33 µg/ml of phleomycin, previously added directly to agar tempered at 50 ˚C. When the hygromycin-resistant transformants were prepared (transformation performed with linearized p434 vector), after the transformation procedure, 625 µl of the cell solution was mixed with 20 ml of antibiotic-free BII regeneration agar, followed by overlaying with 10 ml of BII regeneration agar containing 1.5 mg/ml of hygromycin after 12 hours.

The occurrence of antibiotic-resistant colonies was detected after 4-7 days of

incubation at 28 ˚C.

The colonies were re-inoculated on selection BII agar (pH8, no Fe^{2+}), containing 100 µg/ml of phleomycin or 500 µg/ml of hygromycin, respectively. The growing colonies were used for DNA isolation and their parts were stored as water stocks at 4 °C.

The verified transformants passed the monosporic isolation for obtaining the homokaryotic culture.

3.3.6. DNA isolation and verification of transgene presence

DNA was isolated according to Cenis, 1992.

The presence of a transgene in the genome of transformants was verified by PCR analysis with the PHLEOres_fw, PHLEO res_rev and HYGROrt_fw, HYGROrt_rev primers listed in Table 7, and the number of integrated copies of the transgene by Southern blot analysis; the probes were prepared according to manufactures recommendation (DIG RNA Labeling Kit, Roche, Mannheim, Germany) from the *easC*-pDrive (*easC* gene was amplified with easCfull_fw, easCfull_rev primers) for p444-*easC* presence and copy number verification and from the phleo-pDrive (phleomycin resistance gene amplified with PHLEOres_fw, PHLEO res_rev primers) for p444-*easG* presence and copy number verification Primers are listed in Table 7.

The functionality of the vector to induce strong constitutive expression of the gene of interest was verified by Northern blot analysis (p444-*easG,* p444-*easC*), the probes were prepared with the EasG_full_fw, EasG_full_rev and easCfull_fw, easCfull_rev primers listed in Table 7, or by real-time PCR (p444-*dmaW*, Bražinová, 2012).

The location of the ∆*LpsA2* cassette was verified (homology recombination verification) with the lpsA2_fw, phleoHi3F2 (left flank integration) and lpsA2_rev, phleo_out_Hefe3 (right flank integration) primers listed in Table 7 and the absence of the wild-type allele was verified with the UTRza_lpsA1-gal404fw and lpsA2_k_zac_ rev primers listed in Table 7; (Fig. 8).

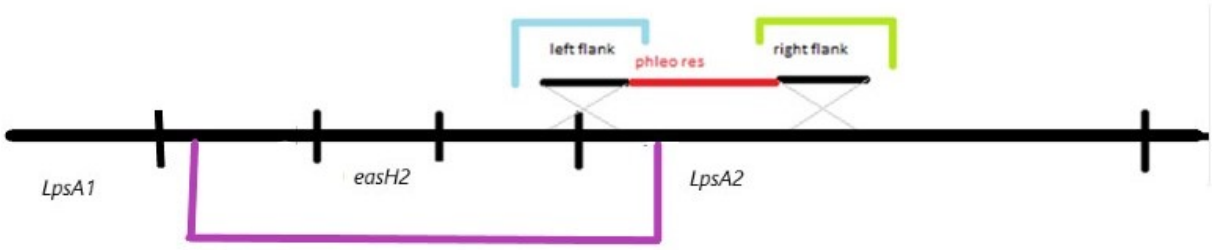


Figure 8. Δ*LpsA2* cassette. The blue line highlights the area amplified for verification of left flank integration; the green line highlights the area amplified for verification of right flank integration. The violet line highlights the area amplified for verification of the presence of the WT allele.

The presence of the OE*LpsA1*_20.1 cassette was verified by PCR, using the Hph fw and LpsA1-sek2_rev primers listed in Table 7.

Diagnostic PCR analyses were performed using the Dreamtaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by standard ethidium bromide-based electrophoreses in 1% agarose gel.

The functionality of pNDH-OGG::lpsA1 gfp- vector was verified by real-time PCR (Hradilová, 2016).

3.3.7. Southern and Northern blot analysis

The chemiluminescence-based method of detection was performed in Southern and Northern blot analysis.

Southern blot analysis was performed with 3-5 µg of EcoRI or ApaI digested, electrophoretically separated DNA in 1% agarose gel. The separated DNA was transferred to a nylon membrane (Whatman Nytran nylon membrane, Sigma-Aldrich) and hybridized with a digoxigenin-labeled probe.

The isolation of the RNA for Northern blot analysis was performed with an RNeasy mini kit (Qiagen). Turbo DNaseI (Thermo Fisher scientific, Waltham, Massachusetts, USA) treated RNA was separated on 1.2% agarose gel containing 0.4M formaldehyde. RNA was transferred to a nylon membrane (Whatman Nytran nylon membrane, Sigma-Aldrich) and hybridized with a digoxigenin-labeled probe.

3.3.8. Inoculation of sterile rye plants

Pollen sterile rye plants *Secale cereale* L. of a cultivar with a high affinity to *C. purpurea* infection (IHAR) were inoculated with verified transformants, as well as with an untransformed culture of the Gal 404 or Gal 130 strain respectively as a wild-type control. The inoculation of the plants was performed during the BBCH62 phenological stage, when approximately 30% of the anthers were mature, by rinsing ears with conidia suspension while puncturing them with a needle.

The infection proceeded in a greenhouse conditions at 20-25 °C/day and 15-18 °C/night. The ears were covered by breathable fabric to avoid cross-infection. The sclerotia were harvested and analyzed qualitatively as well as quantitatively for the production of ergot alkaloids by the HPLC technique.

3.3.9. Isolation of ergot alkaloids and HPLC analysis

The sclerotia were ground in a mortar and weighed and approximately 300 mg of the drug was extracted by means of slow shaking in a rotary shaker in 30 ml of extraction solution (0.3 ml of ammonium hydroxide and 29.7 ml of 90% acetone) for two hours. 10 ml of the extract were pipetted through glass wool and evaporated at 50 °C in a vacuum rotary evaporator. The sample was dissolved in 1 ml of 90% methanol and filtered through a 0.22-mm nylon filter and used for the HPLC analysis of the EA content on a Nexera system (Shimadzu, Kyoto, Japan) with a C18 reverse-phase column (Zorbax RRHD Eclipse Plus, 1.8 mm, 2.1 mm ID x 50 mm, Agilent, Santa Clara, CA, USA).

0.001% (w/v) of each of the following alkaloids: ergotamine, ergostine, 8-hydroxyergotamine, ergocornine, α-ergokryptine, β-ergokryptine, ergocristine, ergogaline, ergotaminine, ergostinine, ergocorninine, α-ergokryptinine, β-ergokryptinine, ergogalinine and ergocristinine in 90% methanol (all from Teva Czech Industries, Opava-Komárov, Czech Republic) was used as the standard for the HPLC analysis. The analytes were eluted with solvents A (36 mM triethylamine/phosphate, pH 4.4, and acetonitrile, 4:1, v/v) and B (H_2O and acetonitrile, 1:4, v/v) using the following gradient: 0 min, 9% B; 10-12 min, 9-13% B; 12-14 min, 13-29% B; 14-16 min, 29-44% B; 18-20 min, 44-59% B; 26-28 min, 59-9% B, at a flow rate of 0.4 ml/min and a column temperature of 30°C. Monitoring

was performed at 317 nm.

3.4 Results and discussion

3.4.1. Introduction of optimized method of transformation for Gal 404 and Gal 130 industrial strains

The optimized method introduced and described above provided about 100-200 colonies per transformation if applied to the Gal 404 and Gal 130 strains.

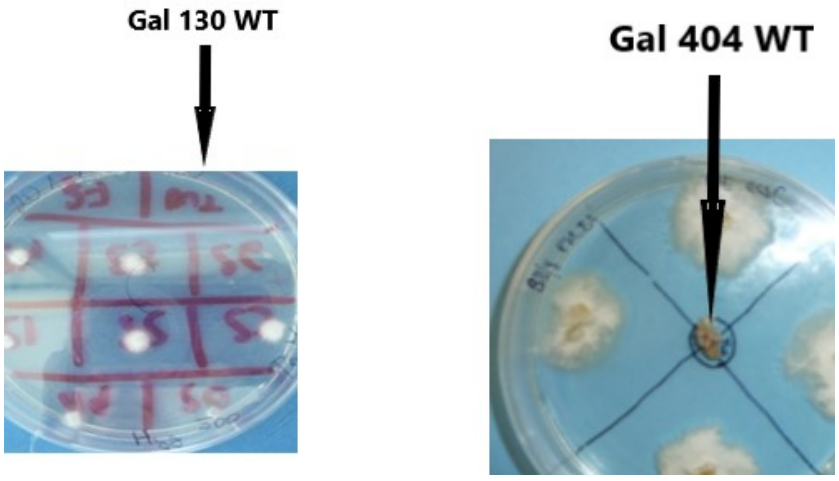


Figure 9. Transformants after monosporic isolation, growing on selection agar with 500 µg/ml hygromycin – Gal130 (A) or 100 µg/ml phleomycin - Gal 404 (B). WT inoculated as selection control.

The introduction of a reliable method for the transformation of an organism of interest is essential “know-how” for reverse genetic research. In many research methods, such as recombinant homology-based knocking-out of a gene, it is necessary to screen large numbers of transformants to get the desired transformants and valuable data. An optimized method for the production of large numbers of transformants per transformation (i.e., up to 200 transformants per transformation) was introduced in the workplace of Department of Molecular Biology, Centre of region Haná, Palacký University with the kind help of Prof. Tudzynski’s group (Fig. 9).

3.4.2. Generation of two independent Gal404:Δ*LpsA1* mutants

In order to describe the substrate specificity of the LPSA1 enzyme present in Gal 404, the ergotamine producer, two independent *LpsA2* knockout mutants were prepared. In total, 230 transformants were screened to obtain these two positive knock-outs meaning that efficiency of homologous recombination was 0,87%.

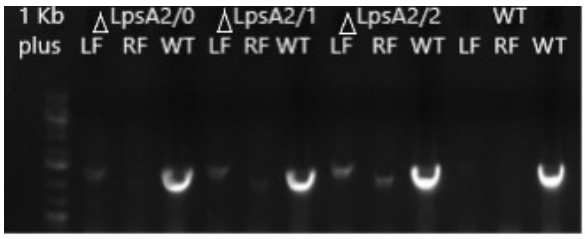


Figure 10. Transformants selected for the presence of homologous recombination before monosporic isolation. LF – lpsA2_fw and phleoHi3F2 primers used for left flank integration verification, RF –lpsA2_rev and phleo_out_Hefe3 primers used for right flank integration verification, WT – UTRza_lpsA1-gal404fw and lpsA2_k_zac_rev2 primers used for verification of the presence of a WT allele.

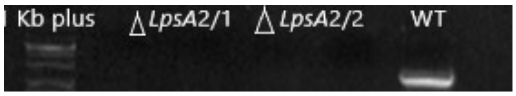


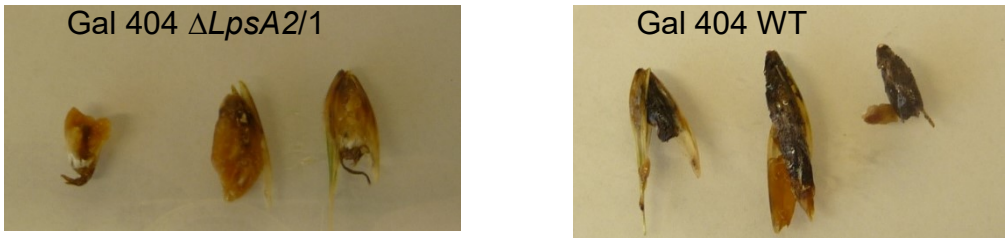
Figure 11. Absence of wild-type allele from knockout mutants after monosporic isolation. The UTRza_lpsA1-gal404fw and lpsA2_k_zac_rev2 primers used for verification of the absence of a WT allele from the knockout mutants.

The verified knockout mutants (Fig. 10 and Fig. 11), as well as the transformant with an ectopically inserted deletion cassette as a control, were inoculated on rye plants and the sclerotia thus formed were tested for changes in their production of ergot alkaloids.

Unfortunately, the analysis of the ergot alkaloids of the knockout sclerotia showed degeneration of the high-producing strain, resulting in a loss of the ability to produce ergot alkaloids in the knockout mutants, as well as in the transformants with an ectopically integrated knock-out cassette. The wild-type strain sclerotia

which grew simultaneously produced a normal range of alkaloids typical of the strain being tested.

The degenerated culture had a reduced ability to infect plants. The production of honeydew was delayed by about two weeks, and the mass of the sclerotia decreased by up to 90%. The sclerotia differed from the wild type in their pigmentation (Fig. 12) and density, suggesting a loss of ability to develop mature sclerotia from the spacelial stage.



A B

Figure 12. Changes in the phenotype of the degenerated Gal 404 Δ*LpsA2*/1 mutant (A) compared with the wild-type Gal 404 strain (B).

The degeneration of strains is a common problem complicating commercial production, as well as research and development of the production of ergot alkaloids (Chen et al., 2017). The combination of the necessary maintenance breeding and the process of transformation is difficult and it is always necessary to consider this option during the data interpretation.

Nevertheless, with the example of the Gal 404:Δ*LpsA2* mutant, we demonstrated that all the necessary steps for the preparation of the knockout mutants of the high-producing strain Gal 404 were introduced and can be used in future for preparation of knock-out strain of any kind of gene from *Claviceps* genome.

3.4.3. Strong constitutive expression of three genes of the EA cluster

Three genes of the ergot alkaloid cluster were constitutively expressed under the control of a strong gpdA promoter in two strains producing large amounts of ergot alkaloids. Diagnostic PCR verified the presence of a transformation cassette (Fig. 13), while Southern blot analysis verified multiple integration of a transgene in most of the tested transformants (see Fig. 14).

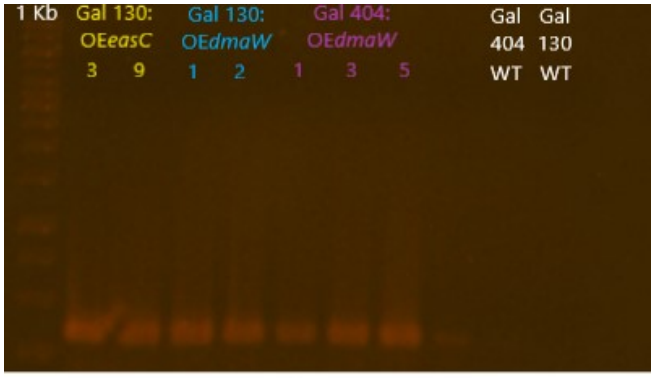


Figure 13. Verification of the presence of phleomycin resistance gene demonstrating successful transformation in Gal130:OE*easC*3, Gal130:OE*easC*9, Gal130:OE*dmaW*1, Gal130:OE*dmaW*2, Gal404:OE*dmaW*1, Gal404:OE*dmaW*3, and Gal404:OE*dmaW*5 vs. absence of phleomycin resistance gene in WT. Transformant number is indicated above the well.

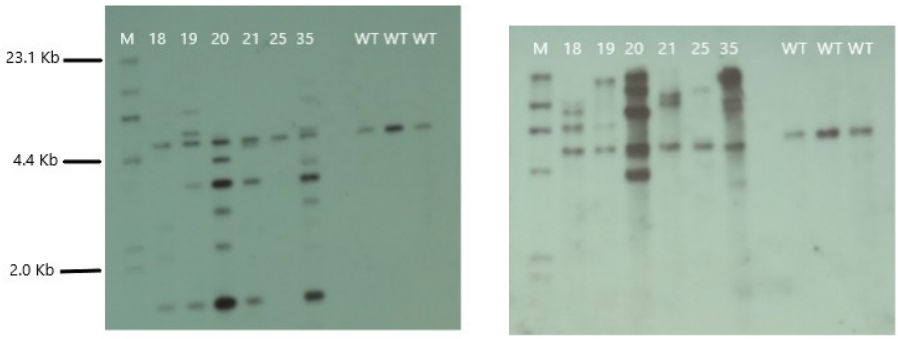


Figure 14. Southern blot analyses verified transformation and showed multiple gene integration in selected transformants. Transformants Gal 404:OE*easC*18, Gal 404:OE*easC*19, Gal 404:OE*easC*20, Gal 404:OE*easC*21, Gal 404:OE*easC*25 and Gal 404:OE*easC*35 were analysed for presence of transformation cassette, WT strain Gal 404 was used as negative control. Transformants number is indicated above the well. The probe for hybridization was prepared using the easCfull_fw and easCfull_rev primers, listed in Table 7. Genomic DNA used for Southern blot analysis was EcoRI (A) or ApaI (B) digested.

Constitutive expression of a transgene was verified by Northern blot analysis (Gal 404:OE*easG*, Gal 404:OE*easC*) or by real-time PCR (Gal 130:OE*dmaW*, Bražinová, 2012) with RNA isolated from mycelia, where the genes of the EA cluster are not expressed or show very low expression (Fig. 15).

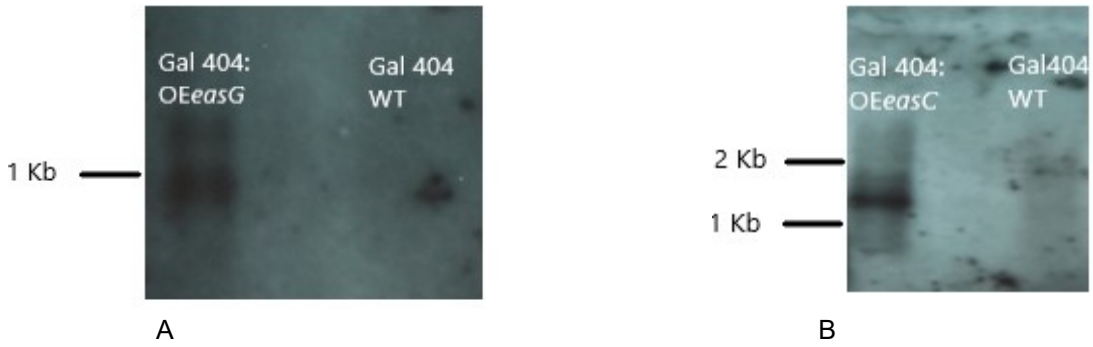


Figure 15. Northern blot analysis of Gal 404:OE*easG* (A) and Gal 404:OE*easC* (B) transformants. The probes for hybridization were prepared using the easCfull_fw and easCfull_rev and EasG_full_fw and EasG_full_rev primers respectively. Primers for probes preparation are listed in Table 7.

The first greenhouse inoculation experiment was performed with four independent transformants of Gal 404:OE*easC* and three independent transformants of Gal 404:OE*easG*. Sclerotia were harvested at a mature stage, i.e., 62 or 69 dpi, and divided into three groups according to their size (large means above 3 cm long, which usually resulted from primary infection, medium from 1.5 to 3 cm and small with length below 1.5 cm). The data from the experiment are summarized in Tables 8 and 9.

	Sclerotia size	**EA content (%)**		**Sclerotia size**	**EA content (%)**
Plant 1, 69 dpi Gal404:OE*easG*2	Small	1.93	**Plant 1, 69 dpi Gal404 WT**	Small	1.34
	Medium	2.12		Medium	1.45
	Large	1.85		Large	1.45
Plant 2, 62 dpi Gal404:OE*easG*2	Small	2.27	**Plant 2, 69 dpi Gal404 WT**	Small	1.45
	Medium	2.35		Medium	1.27
	Large	2.45		Large	1.92
Plant 1, 69 dpi Gal404:OE*easG*3	Small	1.41	**Plant 3, 62 dpi Gal404 WT**	Small	1.54
	Medium	1.21		Medium	1.61
	Large	1.55		Large	1.62
Plant 1, 69 dpi Gal404:OE*easG*4	Small	1.53			
	Medium	1.10			
	Large	1.69			

Table 8. EA content in mature sclerotia of Gal 404:OE*easG* transformants vs. Gal 404 WT expressed as an amount of EA per mass of sclerotia.

	Sclerotia size	Total EA content (%)		Sclerotia size	Total EA content (%)
Plant 1, 69 dpi Gal404:OE*easC* 18	Small	1.34	**Plant 1, 69 dpi Gal404 WT**	Small	1.34
	Medium	1.45		Medium	1.45
Plant 2, 62 dpi Gal404:OE*easC* 18	Small	1.18		Large	1.45
	Medium	2.31	**Plant 2, 69 dpi Gal404 WT**	Small	1.45
	Large	1.25		Medium	1.27
Plant 1, 69 dpi Gal404:OE*easC* 19	Small	0.94		Large	1.92
	Medium	1.09	**Plant 3, 62 dpi Gal404 WT**	Small	1.54
Plant 1, 69 dpi Gal404:OE*easC* 20	Small	1.15		Medium	1.61
	Medium	1.18		Large	1.62
	Large	1.24			
Plant 1, 69 dpi Gal404:OE*easC* 21	Small	1.49			
	Medium	1.46			
	Large	2.23			

Table 9. EA content in mature sclerotia of Gal 404:OE*easC* transformants vs. Gal 404 WT expressed as an amount of EA per mass of sclerota.

A second greenhouse experiment was performed with Gal 404:OE*dmaW* (three independent transformants), Gal 130:OE*dmaW* (one transformant) and Gal 130:OE*easC* (two independent transformants) and with inoculum prepared from Gal 404:OE*easG*2 sclerotia from the first experiment.

Sclerotia were harvested at two time points and HPLC tested for their content of ergot alkaloids (Tab. 10 and 11).

	30dpi, total EA content (%)	55dpi, total EA content (%)		30dpi, total EA content (%)	55dpi, total EA content (%)
Plant1 Gal404 WT	0.23	0.20	**Plant1 Gal 130 WT**	0.25	0.59
Plant2 Gal404 WT	0.26	1.05	**Plant 2 Gal 130 WT**	0.42	0.30
Plant1 Gal404:OE*dmaW*1	0.62	0.54	**Plant1 Gal130:OE*dmaW*2**	0.22	1.05
Plant1 Gal404:OE*dmaW*3	0.49	1.07	**Plant2 Gal130:OE*dmaW*2**	0.01	0.19
Plant2 Gal404:OE*dmaW*3	1.57	1.61			
Plant1 Gal404:OE*dmaW5*	0.18	0.08			

Table 10. EA production in Gal 404:OE*dmaW* vs. EA production in Gal 404 WT in sclerotia harvested at two time points, i.e., 30 and 55 days post-infection respectively. EA content is expressed as an amount of EA per mass of sclerotia.

	30dpi, total EA content (%)	55dpi, total EA content (%)		30dpi, total EA content (%)	55dpi, total EA content (%)
Plant1 Gal 404 WT	0.23	0.20	**Plant1 Gal130 WT**	0.25	0.59
Plant2 Gal 404 WT	0.26	1.05	**Plant2 Gal130 WT**	0.42	0.30
Plant1 Gal404:OE*easG*2	0.71	X	**Plant1 Gal130:OE*easC*3**	0.53	0.57
Plant2 Gal404:OE*easG*2	1.81	1.88	**Plant1 Gal130:OE*easC*9**	0.52	0.88

Table 11. EA production in Gal 404:OE*easG* vs. EA production in Gal 404 WT and EA production in Gal 130:OE*easC* vs. EA production in Gal 130 WT in sclerotia harvested at two time points, i.e. 30 and 55 days post-infection respectively. EA content is expressed as an amount of EA per mass of sclerotia.

Two transformants, i.e., Gal 404:*dmaW*3 and Gal 404:*easG*2, were inoculated on new rye plants in order to test higher production of ergot alkaloids at an early stage, evident in the results from the first time point of the second greenhouse experiment. The time points for the harvesting of the sclerotia were moved to earlier stages, i.e., 20 and 30 dpi (Tab. 12).

	20 dpi, total EA content (%)	30 dpi, total EA content (%)
Plant1 Gal 404 WT	0.18	0.54
Plant2 Gal 404 WT	0.37	0.42
Plant1 Gal 404:OE*easG*2	0.32	0.38
Plant2 Gal 404:OE*easG*2	0.30	0.26
Plant1 Gal 404:OE*dmaW*3	0.18	0.26
Plant2 Gal 404:OE*dmaW*3	0.14	0.30
Plant3 Gal 404:OE*dmaW*3	0.09	0.82

Table 12. EA production in Gal 404:OE*easG*2 and Gal 404:OE*dmaW*3 vs. EA production in Gal 404 WT in sclerotia harvested at two time points, i.e., 20 and 30 days post-infection respectively. EA content is expressed as an amount of EA per mass of sclerotia.

The sclerotia of the first greenhouse experiment were harvested at a mature stage. Gal 404:OE*easG*2 was selected for further testing because of its higher content of ergot alkaloids when compared with simultaneously grown Gal 404 WT sclerotia. The level of alkaloids produced in Gal 404:OE*easG*2 was up to 10%

higher than the upper limit typical of the strain, described in Majeska, 2016.

The sclerotia of the industrial strains Gal 404 and Gal 130 show significant diversity in their levels of production of ergot alkaloids. The production of WT strains oscillates between 0.4% to 2.2% in Gal 404 and 0.73% to 2.66% in Gal 130 respectively (Majeska et al., 2016).Deviations in production are caused by natural diversity and the improbability of choosing sclerotia of the same age, as part of them come from primary infection and part of them from secondary infection induced by honeydew production.

The sclerotia of the second greenhouse experiment were harvested at two time points, i.e., 30 and 55 dpi. The data from the second greenhouse experiment suggested higher production in the early stage of some transformants (Gal 404:OE*dmaW*3 and Gal 404:OE*easG*2; Tab. 8 and 9), suggesting the effect of overexpression in the early stage, where naturally occurring genes connected with the production of ergot alkaloids are expressed weaker.

The mature sclerotia of the transformants produced higher amounts of alkaloids, if compared with simultaneously grown WT strains, but did not exceed the usual level of production that is typical of the strains.

The experiment was repeated with Gal 404:OE*dmaW*3 and Gal 404:OE*easG2*, with the time points of the harvesting of the sclerotia being moved to earlier stages, i.e., 20 and 30 dpi. The data acquired showed the similar or lower production of ergot alkaloids in both transformants compared to the WT strain at both monitored time points, contrary to the first results.

The data suggest that none of genes selected for overexpression in industrial strains is a significant limiting factor for the production of ergot alkaloids in these infectious, strongly ergot alkaloid-producing strains. Although the limiting factors may vary in different strains, especially among strains producing alkaloids in submerged culture and infectious strains.

The weakening of the transformant production in repetitions of the experiment can also be caused by failure in maintenance breeding in the limited conditions of a greenhouse.

3.4.4. Heterologous constitutive expression of *LpsA1* gene in Gal 130

The *LpsA1* gene, responsible for the production of ergotamine in the 20.1 strain (Haarmann et al., 2008), was constitutively expressed in the Gal 130 strain, the ergocristine producer. The transformation was verified by diagnostic PCR with the hph fw and LpsA1-sek2_rev primers listed in Table 7 (Fig. 16).

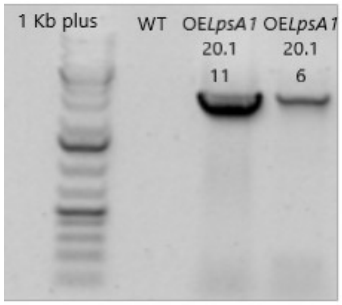


Figure 16. Diagnostic PCR verification of transformation in two independent transformants, i.e., Gal130:OE*LpsA1*, 20.1/11 and Gal130:OE*LpsA1*, 20.1/6. The primers used for the verification of the integration of the transformation cassette are hph fw and LpsA1-sek2_rev, listed in Table 7.

Two independent transformants were inoculated on rye plants and sclerotia harvested at three time points were analyzed for changes in their ergot alkaloid spectra (Tab. 13).

19 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	91.0	94.9	95.8
	αErgokryptine+inine	2.6	0.0	3.1
	Ergotamine+inine	6.4	5.1	1.1
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	90.3	95.8	98.1
	αErgokryptine+inine	2.7	0.0	1.7
	Ergotamine+inine	7.0	4.2	0.2
	3. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	91.7	89.1	96.0
	αErgokryptine+inine	2.4	1.9	3.0
	Ergotamine+inine	5.9	9.0	1.0
31 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.3	88.4	96.0
	αErgokryptine+inine	2.9	4.5	3.0
	Ergotamine+inine	2.8	7.1	1.0
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.2	94.5	96.6
	αErgokryptine+inine	2.9	2.6	2.7
	Ergotamine+inine	2.9	2.9	0.6
55 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	93.0	91.0	95.0
	αErgokryptine+inine	4.1	0.0	3.5
	Ergotamine+inine	2.9	9.0	1.4
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	93.8	90.3	94.8
	αErgokryptine+inine	3.6	2.9	3.9
	Ergotamine+inine	2.6	6.7	1.3
	3. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.8	71.4	95.6
	αErgokryptine+inine	3.0	0.0	3.1
	Ergotamine+inine	2.2	28.6	1.2

Table 13. Changes in relative ergotamine representation in EA pool of two independent transformants Gal 130:OE*LpsA*1,20.1 compared to Gal 130 WT.

Significant qualitative changes in the EA spectra of two independent Gal 130:OE*LpsA1*, 20.1 transformants were monitored at three time points during infection. The effect of heterologically expressed *LpsA1*, and therefore changes in ergotamine representation, were significant mainly in the early stages of the development of the sclerotia, when the expression of naturally occurring ergot alkaloid cluster genes is lower. Affecting the ergot alkaloid spectra by heterologous *LpsA* expression could open new possibilities for the designing of new *C. purpurea* production strains, although the size of the gene (i.e., approx. 11 kb) can delimitate the level of expression.

Using a suitable, strong promoter, the heterologous expression of *LpsA* can lead, e.g., to the preparation of submerged producing strains with altered EA spectra, although the supposed effect of the actual concentration of the relevant amino acid

on the type of produced EA in the strain must be considered.

3.5 Conclusion

A method for *C. purpurea* transformation providing a large number of transformants per transformation was successfully introduced.

The method was used for the preparation of two independent Gal404:Δ*LpsA2* mutants in order to determine the substrate specificity of the LPSA1 and LPSA2 enzymes by monitoring changes in the EA spectra and focusing on both the major and minor alkaloids that are produced. Strain degeneration resulting in total loss of EA production made evaluation of the experiment impossible.

Three genes of the ergot alkaloid pathway, i.e., *dmaW*, *easC* and *easG*, were constitutively expressed in the Gal 404 and/or Gal 130 strains under the control of a strong gpdA promoter. The mutants were screened for changes in their total production of alkaloids. No biotechnologically significant, sustainable changes in EA production were observed.

The *LpsA1* gene originating from the 20.1 strain, the ergotamine producer, was constitutively expressed in the Gal 130 strain, the ergocristine producer. Two independent Gal 130:OE*LpsA1*, 20.1 mutants were monitored for changes in ergotamine representation in the EA pool at three time points during infection. A significant improvement in ergotamine representation up to 28% of total EA content, which is originally the minor alkaloid of the strain, representing in WT only around 1% of the EA pool, was observed in both mutants.

Primer name	Sequence (5´-3´)
EasG_full_fw	CCGGAATTCATGACGGTCTTACTGACAGGAGG
EasG_full_rev	CGGGGTACCTCACTTTCTTGCACGCCAG
easCfull_fw	ATGGCTTCTGAGGTCTCTG
easCfull_rev	TTACTCCACTATCTCTTCGG
DMaWfull_fw	ATGTCGACCGCAAAGGACCCAG
DMaWfull_rev	CTACTTYGTKGAGAGKTCACAGCG
PHLEOres_fw	GACTCCGTGGAGGACGACTT
PHLEOres_rev	TGCTCGCCGATCTCGGTCAT
HYGROrt_fw	CGAGGTCGCCAACATCTTCT
HYGROrt_rev	GCGTCTGCTGCTCCATACAA
UTRza_lpsA1-gal404fw	AGGCCGATACTGCGTTAC
lpsA2_k_zac_rev2	TTCCGAGAACTACAGCCCA
Hph fw	CCTCCACTAGCTCCAGCCAAGCCC
LpsA1-sek2_rev	AGCTTCACAACAAATGGCGT
5F_lpsA2_GAL404	CCAGGGTTTTCCCAGTCACGACGGGATCCCTGGACGGGCAATCAATCAG
5R_lpsA2_GAL404	CCACTTAACGTTACTGAAATCTCCAACCAGCAGAGCCGGACGATATA
3F_lpsA2_GAL404	CTCCTTCAATATCATCTTCTGTCTCCGACTGTGGTCAACGCCGAGAGTA
3R_lpsA2_GAL404	ACAATTTCACACAGGAAACAGCGGATCCTGGAGAGTCTGGGCATCTAAC
dia_lpsA2_GAL404_fw	CACCGACAAGTTGTGGACAC
dia_lpsA2_GAL404_rev	TAATAACTGGAGGCCCTATAG
phleo_Hi3F2	GTGTTCAGGATCTCGATAAGATACG
Phleo_out_Hefe3	GAGCTCGGTATAAGCTCTCC
CpBleF1	CGGAGACAGAAGATGATATTGAAGGAGCGATCGAGACCTAATACAGCCCC
CpBleR1	GTTGGAGATTTCAGTAACGTTAAGTGGGCATTGCAGATGAGCTGTATCTG

Table 7. Oligonucleotide primers used in the study

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5 Abbreviations

BBCH-scale – scale used to identify the phonological development stages of a plant
ccsA – gene encoding chanoclavine synthase from *Claviceps purpurea*
cdc42 – gene encoding GTPase from *Claviceps purpurea*
cla4 – gene encoding P21-activated kinase from *Claviceps purpurea*
cloA – gene encoding P450 monooxygenase catalyzing conversion of agroclavine to elymoclavine and subsequently D-Lysergic acid
cloA – P450 monooxygenase catalyzing conversion of agroclavine to elymoclavine and subsequently D-Lysergic acid
cot1 – gene encoding Ser/Thr kinase from *Claviceps purpurea*
cpps1 – see *lpsA*
CPPS1 – see LPSA
cpps3 – see *lpsC*
cpps4 – see *LpsA*
DMAPP – dimethylallylpyrophosphate
DMAT - 4-(γ,γ-dimethylallyl)tryptophan
dmaW – gene encoding dimethylallyltryptophan synthase from *Claviceps purpurea*
dpi – days post infection
EA – ergot alkaloids
EAS- ergot alkaloid syntheses
easC – gene encoding chanoclavine synthase catalase protein from *Claviceps purpurea*
easC – chanoclavine synthase catalase protein from *Claviceps purpurea*
easD – gene encoding chanoclavine-I dehydrogenase from *Claviceps purpurea*
easD – chanoclavine-I dehydrogenase from *Claviceps purpurea*
easE – gene encoding chanoclavine-I-synthase oxidoreductase from *Claviceps purpurea*
easE – chanoclavine-I-synthase oxidoreductase from *Claviceps purpurea*
easF – gene encoding 4-dimethylallyltryptophan N-methyltransferase from *Claviceps purpurea*
easF – 4-dimethylallyltryptophan N-methyltransferase from *Claviceps purpurea*
easG – gene encoding agroclavine synthese from *Claviceps purpurea*
easG – agroclavine synthese from *Claviceps purpurea*
easH – gene encoding dioxygenase from *Claviceps purpurea*
easH – dioxygenase from *Claviceps purpurea*
ETH – ethrel, 2-chloroethylphosphonic acid
FAD – flavin adenine dinucleotide
GAPDH – glycerol-3-phosphate dehydrogenase
gpdA – promoter for glyceraldehyde-3-phosphate dehydrogenase from *Aspergillus nidulans*
Hk2 – gene encoding His kinase from *Claviceps purpurea*
Hph – gene encoding hygromycin B phosphotransferase
LaeA – gene encoding protein that is part of a Velvet complex in fungi

LPSA - D-Lysergyl-peptide synthetase subunit 1

LpsA – gene encoding D-Lysergyl-peptide synthetase subunit 1

LPSB – D-Lysergyl-peptide synthetase subunit 2

LpsB – gene encoding D-Lysergyl-peptide synthetase subunit 2

LPSC – Alanine-activating Subunit of Ergometrine Synthetase

LpsC – gene encoding Alanine-activating Subunit of Ergometrine Synthetase

Lps3 – see *lpsC*

LPS3 – see LPSC

Lps4 – see *LpsA*

LPS4 – see LPSA

LSD – lysergic acid diethylamide

MeDMAT – *N*-methyl-dimethylallyltryptophan

MH – maleic hydrazide; 1,2-dihydro-3,6-pyridazinedione

Mid1 – gene encoding protein Ca^{2+} channel from *Claviceps purpurea*

Mk ½ – gene encoding MAP kinase from *Claviceps purpurea*

NADPH – nicotinamide adenine dinucleotide phosphate

Nox1 – gene encoding NADPH-oxidase from *Claviceps purpurea*

NRPS – nonribosomal peptide-synthetase

ORF – open reading frame

PEG – Polyethylenglycol

Pg ½ – gene encoding polygalacturonase from *Claviceps purpurea*

poliC – promoter for ATPase subunit 9 from *Aspergillus nidulans*

Rac – gene encoding GTPase from *Claviceps purpurea*

RT – room temperature

ShBle – gene encoding bleomycin resistance protein from *Streptoalloteichus hindustanus*

trpC – terminator for indole-3-glycerol phosphate synthese from *Aspergullus nidulans*

Tf1 – gene encoding CREB-like transcriptional factor from *Claviceps purpurea*

WT – wild type

Xyl2 – gene encoding endo-1,4-β-xylanase from *Claviceps purpurea*

5-HTs – 5-hydroxytryptamine receptor

6 Curriculum vitae

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MSc. Biochemistry

- Research project: Genetic modification of *Claviceps purpurea*

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Teva Pharmaceutical Industries Ltd, Hungary

- Training in *Claviceps* fermentation techniques

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- Training in *Claviceps purpurea* transformation techniques

August 2013

University of Muenster, Germany

- *LpsA2* knock-out mutants generation

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- Testing of susceptibility of cultivars of wheat and rye to *Claviceps purpurea* infection

TEACHING

2010 - KBC/LT Basic laboratory technique for biochemists

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Hanosová H, Koprna R, Valík J, Knoppová L, Frébort I, Dzurová L, Galuszka P. Improving field production of ergot alkaloids by application of gametocide on rye host plants. N Biotechnol. 2015, 32(6):739-46.

SCIENTIFIC CONFERENCES

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7 Supplements

Supplement 7.1

Review article

Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids

Helena Hanosová (Hulvová), Petr Galuszka, Jitka Frébortová, Ivo Frébort

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Research review paper

Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids

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ABSTRACT

Ergot alkaloids produced by the fungus *Claviceps* parasitizing on cereals, include three major groups: clavine alkaloids, D-lysergic acid and its derivatives and ergopeptines. These alkaloids are important substances for the pharmatech industry, where they are used for production of anti-migraine drugs, uterotonics, prolactin inhibitors, anti-Parkinson agents, etc. Production of ergot alkaloids is based either on traditional field cultivation of ergot-infected rye or on submerged cultures of the fungus in industrial fermentation plants. In 2010, the total production of these alkaloids in the world was about 20,000 kg, of which field cultivation contributed about 50%. This review covers the recent advances in understanding of the genetics and regulation of biosynthesis of ergot alkaloids, focusing on possible applications of the new knowledge to improve the production yield.

Contents

1. Introduction

The *Clavicipitaceae* family includes, among others, a fungal species called ergot that is important for humankind, in terms of both contributions and losses. In medieval history, these fungi parasitizing in cereals and producing a range of ergot alkaloids caused many mass poisonings that resulted in painful deaths of tens of thousands of people (Schiff, 2006). Nowadays, mass poisonings with ergot alkaloids are sporadic and occur only in developing countries. For example, an epidemic of ergotism with a high mortality rate occurred in 1977 in Ethiopia (Demeke et al., 1979). Contrary to mass poisonings, the recent cases of individual poisonings are usually connected with overdoses of medical drugs based on these alkaloids.

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For thousands of years, people tried to take advantage of substances produced by ergot without knowing their chemical structure, proper dosage or side effects, but scientific research on ergot alkaloids started only with the beginning of modern pharmacy in the early 20th century. Nowadays, specific types of ergot alkaloids are widely used as a basic drug-stock for the production of various therapeutic substances, e.g. for the treatment of migraine, in gynecology for its uterotonic effects, as prolactin inhibitors, antiparkinsonian drugs, etc. (de Groot et al., 1998).

The *Claviceps* genus is not only interesting for its ability to produce secondary metabolites usable in pharmaceutical industry, but also in its life strategy, mainly the specificity of invaded host organs, which is in the center of interest of many scientific groups. Last, but not least, its negative influence in agriculture should be mentioned. Some of the *Claviceps* species cause significant losses in cereal production. Recently, there was an infection of the fifth most important cereal crop in the world, *Sorghum bicolor* (Haarmann et al., 2009), by *Claviceps africana*.

2. Ergot alkaloids

2.1. History of poisoning and use of ergot alkaloids

First references to ergot use can be found in early history. The oldest documentation of the positive effects of ergot alkaloids in obstetrics appeared in China in approximately 1100 BC (Schiff, 2006). In one of the sacred books of the Parsees (400 BC to 300 BC), ergot is mentioned as "noxious grasses that cause pregnant women to drop the womb and die in childbirth" (Thoms, 1931). In the Middle Ages, there were many documented cases of mass intoxication with ergot caused by contaminated cereals, usually referred to as ergotism. There are two types of ergotism, which differ in symptoms. The first one, called "convulsive ergotism", was typical for the area located east of the Rhine river in Europe and was accompanied by muscle spasms, hallucinations and fever. These symptoms are typical for serotonergic stimulation of the central nervous system, caused by the activation of serotonin receptors by ergot alkaloids, due to their structural similarity to the neurotransmitter serotonin (Eadie, 2003). The second type of ergotism, typical for the area west of Rhine, is called "gangrenous ergotism", and is accompanied by violent burning and shooting pain of the affected acral part of the human body. Related to the saint who suffered horrible visions sent by the devil, this type of ergotism has been called St. Anthony's fire (Lee, 2009). The first documented epidemic of ergotism is dated 944–945 AD and caused the death of about 10,000 people in France. Some 50 years later, intoxication with ergot alkaloids again killed about 40,000 people in this area. It is likely that ergot alkaloid intoxication was also connected with the well-known witch trials of 1692 in Salem, Massachusetts, USA (Caporael, 1976; Spanos and Gottlieb, 1976) and in Finnmark, Norway in the 17th century (Alm, 2003). A correlation between the symptoms of ergotism and ergot consumption was understood finally in the 1850s, due to the findings of Louis René Tulasne, a French mycologist, who first fully described the life cycle of ergot (Tulasne, 1853). Modern ergot alkaloid research started in 1918 with ergotamine isolation by the Swiss biochemist Arthur Stoll (Stoll, 1945). In 1926, Swiss psychiatrist Hans Maier suggested that ergotamine might be useful in the treatment of vascular headaches of the migraine type (Silberstein et al., 2001). LSD, a synthetic derivative of lysergic acid, is one of the components of ergot alkaloid blend that was first synthesized in 1938 by the Swiss chemist, Alfred Hoffman, but its effect on nervous system was not discovered until he accidentally contaminated himself and experienced the hallucinogenic reaction in 1943 (Minghetti and Crespi-Perellino, 1999). The drug became popular in the mid-1960s when its sense-altering properties were reputed to offer a window into enhanced creativity and self-awareness.

2.2. Chemistry and occurrence of ergot alkaloids

Ergot alkaloids belong to the class of indole derivatives. They can be divided into three major groups: clavine alkaloids, D-lysergic acid and its simple derivatives and ergopeptines. Structures of typical ergot alkaloids are shown in Fig. 1. Species within the genus *Claviceps* differ in their capability to produce diverse types of alkaloids. Only a few species (e.g. *Claviceps purpurea* and *C. africana*) can produce ergopeptines as final products of their ergot alkaloid biosynthetic pathway. For example, the biosynthetic pathway of *Claviceps fusiformis* ends with elymoclavine production that has been explained as a loss of the late pathway genes in the ergot alkaloid gene cluster (Lorenz et al., 2007).

Most of ergot alkaloids contain a tetracyclic ergoline structure, although some of the naturally occurring clavine alkaloids are tricyclic, e.g. chanoclavine-I, chanoclavine-II and isochanoclavine-I (for a review see Buchta and Cvak, 1999). Of these, only chanoclavine-I can serve as a precursor for biosynthesis of other ergot alkaloids. Tetracyclic clavine

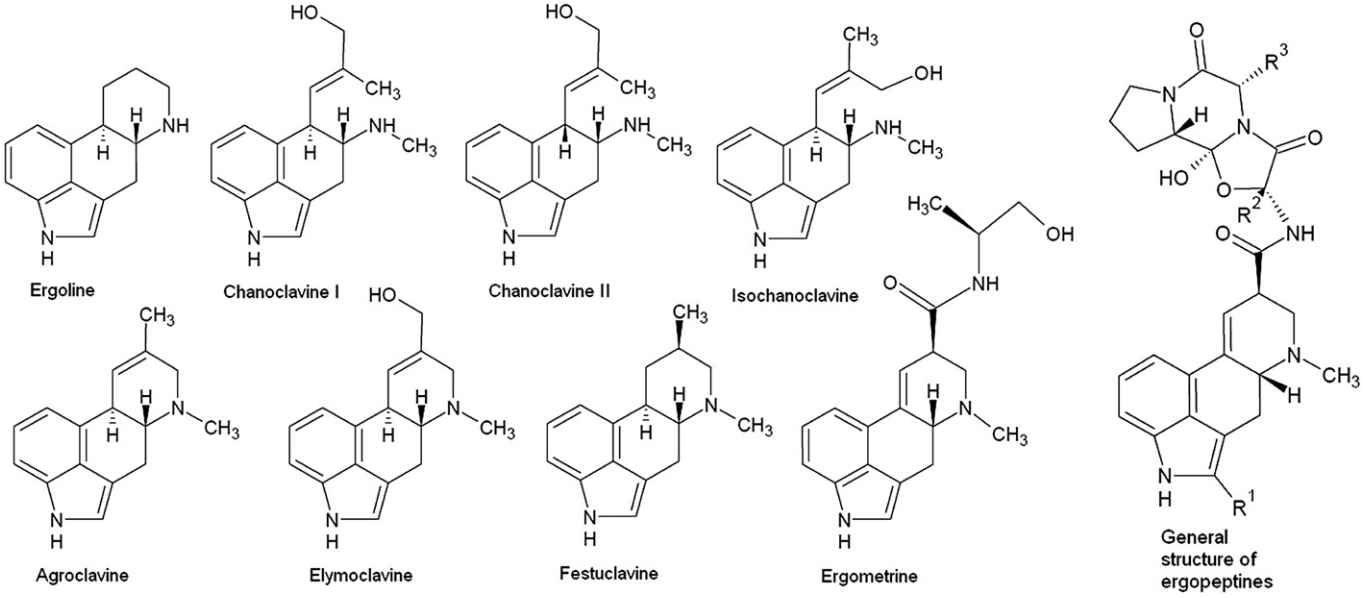

Fig. 1. Structures of ergot alkaloids.

alkaloids can be found in various species of fungi. Agroclavine and elymoclavine are intermediates of the pathway leading to D-lysergic acid production, but in most ergot alkaloid producing fungi, this pathway ends with clavine alkaloids as final products (see Fig. 2A). Besides the genus *Claviceps*, ergot alkaloids were also once reported in other fungi, such as *Botrytis fabae* (Naim, 1980) and *Geotrichum candidum* (El-Refai et al., 1970). However, their occurrence was later unconfirmed by more advanced analytical methods. Similarly, ergopeptines were mentioned to be produced by *Aspergilus fumigatus* (Abe et al., 1967; Cole et al., 1977; Narayan and Rao, 1982; Spilbury and Wilkinson, 1961), but this was not confirmed more recently.

Derivatives of D-lysergic acid can be divided into simple amides and tripeptides. One of the representatives of D-lysergic amides is ergometrine (alternatively also called ergonovine or ergobasine) derived from D-lysergic acid and 2-aminopropanol. Ergometrine is produced by *C. purpurea* together with ergopeptines (Dudley and Moir, 1935), which are built from the lysergic acid fragment and a tripeptide group composed of diverse amino acids, where proline is always in the third position. Ergopeptines are the end products of the biosynthetic pathway of ergot alkaloids. The main producer of ergopeptines is *C. purpurea*, but dihydro-α-ergosine is also produced by *C. africana* (Mantle and Waight, 1968).

The presence of clavine alkaloids and ergopeptines had been for a long time also associated with dicotyledonous plant species of the family *Convolvulaceae*, also known as the bindweed or morning glory. In these plants, the alkaloids are produced by different species of endophytic fungi that are mostly seed-transmitted and may have shared their alkaloid producing genes by hybridization or horizontal transfer (Clay and Schardl, 2002). Microscopic clavicipitalean fungi were recently detected residing on leaves and seeds of *Ipomoea asarifolia* and *Turbina corymbosa* (Steiner et al., 2006). This epibiotic fungi that contains genes involved in ergot alkaloid biosynthesis (Markert et al., 2008) was recently described as a new genus, *Periglandula* (Steiner et al., 2011).

2.3. Biosynthesis of ergot alkaloids: enzymology and genetics

Essential steps of the ergot alkaloid biosynthetic pathway were analyzed by feeding *Claviceps* liquid cultures with isotopically labeled precursors. The general biosynthetic pathway of ergot alkaloids is shown in Fig. 2A,B. As precursors of the ergoline system, three primary metabolites were found: tryptophan, a product of the mevalonate pathway dimethylallylpyrophosphate (DMAPP) as a donor of the isoprene unit, and methionine as a methyl group donor. The first specific step of the biosynthetic pathway is the isoprenylation of tryptophan resulting in 4-(γ,γ-dimethylallyl)tryptophan (DMAT). This step, catalyzed by 105 kDa homodimer enzyme DMAT synthase (Gebler and Poulter, 1992), is positively regulated by tryptophan and negatively feedback-regulated by agroclavine and elymoclavine, further products of the biosynthetic pathway (Cheng et al., 1980). *DmaW* gene coding for DMAT synthase, originally cloned and sequenced from *C. fusiformis*, was the first discovered gene of the ergot alkaloid synthesis (EAS) cluster (Tsai et al., 1995). The 1517 bp ortholog of *dmaW* containing two introns was found in *C. purpurea*, proving a common biosynthetic

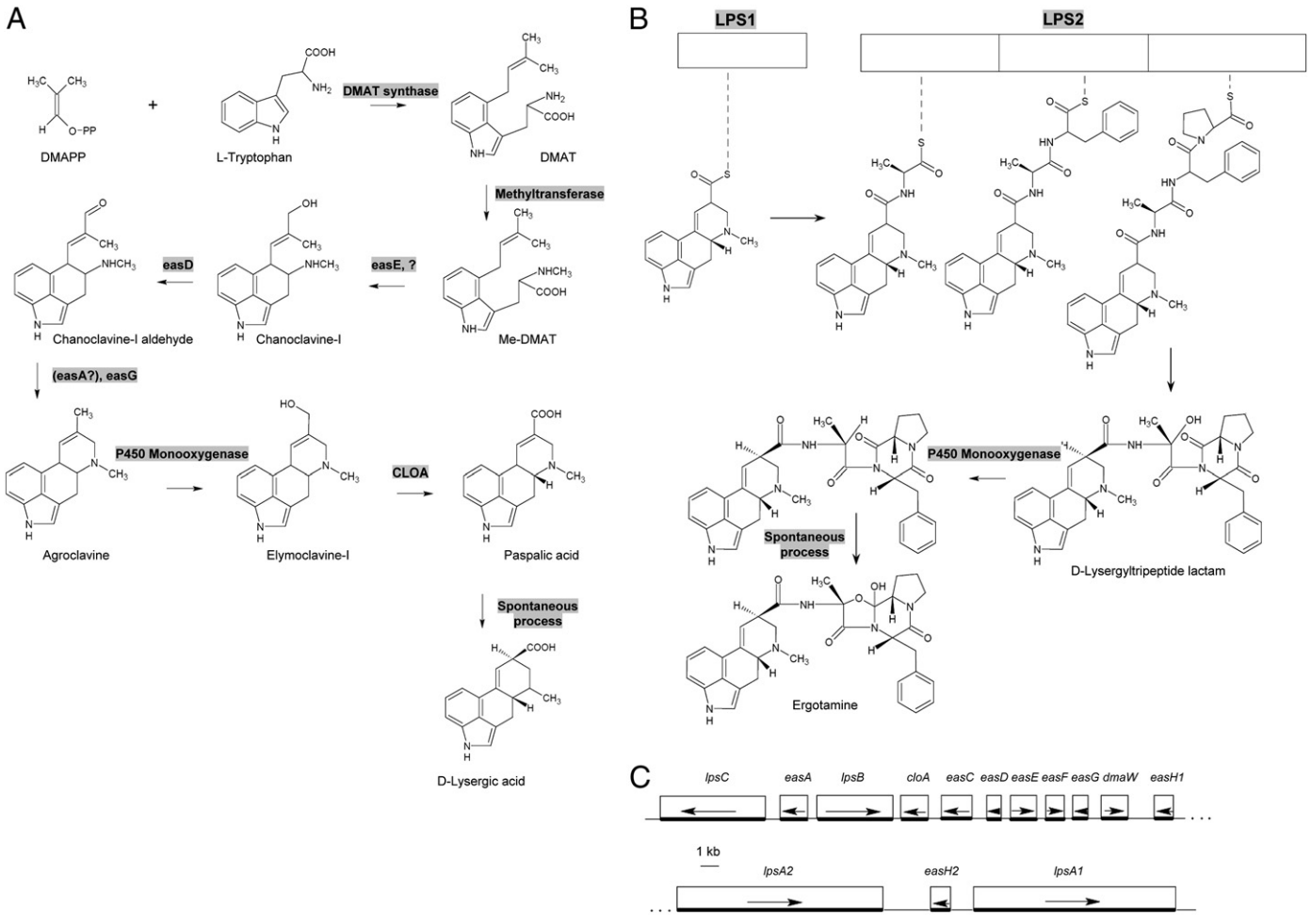

Fig. 2. Biosynthesis of ergot alkaloids in *Claviceps purpurea*: A) scheme of the biosynthetic pathway leading to D-lysergic acid, B) biosynthesis of ergopeptines on two-component non-ribosomal peptide synthetase (NRPS), and C) scheme of the ergot alkaloid synthesis (EAS) gene cluster.

origin of clavines and D-lysergic acid-derived alkaloids (Arntz and Tudzynski, 1997). Analyses of 3′ flanking region of *dmaW* showed a presence of another EAS related gene named *cpps1* (Tudzynski et al., 1999). By chromosome walking, a whole 68.5 kb EAS cluster, consisting of 14 genes (Fig. 2C), was described by Paul Tudzynski's group (Haarmann et al., 2005).

The second step of the EAS pathway is the methylation of DMAT to *N*-methyl-dimethylallyltryptophan (MeDMAT). Otsuka et al. (1980) first isolated an enzyme that is responsible for catalyzing the methylation of DMAT using S-adenosylmethionine as a donor of the methyl group. The biosynthetic pathway continues with several oxidation and reduction steps resulting in the formation of chanoclavine I, chanoclavine I aldehyde and then agroclavine. Chanoclavine I is the only one of the four stereoisomers of chanoclavine, which can be further converted into tetracyclic ergolines (Gröger et al., 1966). Chanoclavine I synthase, a FAD-containing oxidoreductase, is involved in the oxidation of MeDMAT to chanoclavine I, but there are still some other enzymes needed to complete the conversion. The mechanism of this reaction was proposed by Gröger and Floss (1998). In *C. purpurea*, chanoclavine synthase is encoded by *ccsA* gene of 1503 bp, originally named *easE*. The coding region of *ccsA* gene is interrupted by one intron (Lorenz et al., 2010). Chanoclavine-I is then further oxidized to chanoclavine-I aldehyde by an alcohol dehydrogenase encoded by *easD* (Wallwey and Li, 2011).

Closure of D ring of tetracyclic ergot alkaloids proceeds via *cis-trans* isomerization on C2 of the mevalonate group (Floss, 2006) in a series of enzymatic steps, collectively called chanoclavine cyclase. Chanoclavine-I aldehyde first undergoes double bond isomerization to isochanoclavine-I aldehyde, originally thought to be catalyzed by the product of *easA* gene. It was shown recently that the reaction also proceeds non-enzymatically by reduction with glutathione or 2-mercaptoethanol (Matuschek et al., 2011). Isochanoclavine-I aldehyde then forms an iminium ion compound, which is subsequently reduced to agroclavine by the enzyme easG (agroclavine synthase) in the presence of NADPH. The monomeric easG of 31.9 kDa consists of 290 amino acids and is encoded by *easG* gene of 1079 bp containing two introns (Matuschek et al., 2011).

The biosynthetic pathway then continues with a P450 monooxygenase catalyzed oxidation of agroclavine on C17 atom, leading to formation of elymoclavine. Elymoclavine is oxidized to paspalic acid by another P450 monooxygenase, clavine oxidase (CLOA). This enzyme connects the formation of two groups of ergot alkaloids, the clavine alkaloids and D-lysergic acid derived alkaloids, such as simple D-lysergic acid amides and ergopeptines. CLOA protein is encoded by *cloA* gene of 2120 bp interrupted with eight introns (Haarmann et al., 2006). Paspalic acid is converted to D-lysergic acid spontaneously, as described by Gröger and Floss (1998).

Elymoclavine monooxygenase and all subsequent enzymes are not encoded in fungal genomes that produce the clavine type of alkaloids. The following metabolic steps lead to amides of D-lysergic acid and ergopeptines, crucial metabolites of *C. purpurea*. D-lysergic acid is captured and activated by monomodular non-ribosomal peptide synthetase (NRPS) LPS2, one of the two subunits of D-lysergyl peptide synthetase with a molecular mass of 141 kDa. D-lysergic acid is, after binding to LPS2 as a thioester, transferred to trimodular LPS1 NRPS, the second subunit of D-lysergyl peptide synthetase with a size of 370 kDa. Here it is condensed with three bound amino acids, resulting in formation of a D-lysergyl tripeptide lactam (Walzel et al., 1997). LPS2 and LPS1 NRPS are encoded by *cpps2* and *cpps1* genes, respectively (Correia et al., 2003; Tudzynski et al., 1999). *Cpps2*, also named *lpsB*, is a gene of 3991 bp containing one intron, while *cpps1*, also named *lpsA*, consists of 10,850 bp and contains two introns.

Finally, D-lysergyl peptide lactam is oxidized by another P450 monooxygenase and the product is spontaneously converted to ergopeptines (Haarmann et al., 2006). The structure of final ergopeptine depends on the amino acids used for the condensation with D-lysergic acid, where the first two positions are occupied by non-polar amino acids and the third one exclusively by proline (Keller, 1999).

C. purpurea can also synthesize D-lysergylalkanolamides, alkylamides of D-lysergic acid, such as ergometrine. In these compounds, D-lysergic acid is linked to an aminoalcohol derived from alanine. The reaction is catalyzed by LPS2 as the D-lysergic acid activator and another monomodular NRPS subunit, ergometrine synthetase, in the presence of NADPH. LPS3 enzyme (ergometrine synthetase) is encoded by *cpps3* gene (also called *lps3*) located in the EAS gene cluster (Ortel and Keller, 2009).

The EAS cluster contains one more NRPS gene, *cpps4* (also called *lps4*), which encodes LPS4 protein with a function similar to LPS1. The two enzymes differ in their capability to bind different amino acids used for ergopeptine formation. LPS4 is responsible for α-ergocryptine production in P1 strain of *C. purpurea*, while LPS1 catalyzes the production of ergotamine (Haarmann et al., 2005).

2.4. Biological activity of ergot alkaloids

Specific types of ergot alkaloids serve as a basic drug-stock for production of various therapeutic substances to treat migraine headaches, Parkinson's disease, hypertension and diverse sexual disorders (de Groot et al., 1998). Biological activity of ergot alkaloids relates to their structural similarities with neurotransmitters noradrenalin, dopamine and serotonin (Fig. 3). The major pharmaceutical effects of ergot alkaloids are smooth muscle stimulation, central sympatholytic activity and peripheral α_1-adrenergic blockade. Smooth muscle stimulation is the most evident as a vasoconstriction and uterine contraction (Innes, 1962). Since the ergot alkaloids are dopamine receptor agonists, some of them (bromocriptine, cabergoline, pergolide) can be used as inhibitors of prolactin release (Nasr and Pearson, 1975) and anti-Parkinson agents. However, they are not recommended as a first-line antiparkinsonian medication because of the risk of fibrotic reaction (Bonuccelli et al., 2009). Effects of the ergot alkaloids on dopamine receptors are described in more detail in Emilien et al. (1999). Anti-migraine effects of many ergot alkaloids are facilitated by interactions with serotonin receptors, 5-HTs. First compound used for the treatment of this disorder was ergotamine in 1926 (Maier, 1926). The main disadvantage of natural ergot alkaloids is a lack of selectivity for each individual 5-HT receptor, which stimulated the development of semisynthetic serotonergic ligands that are more selective (Pertz and Eich, 1999). Interactions of the ergot alkaloids and their derivatives with serotonin receptors are reviewed in Pertz and Eich (1999). Ergot alkaloids also interact with α_1-adrenoceptors, which mediate vascular contractility. Many ergot alkaloids and their derivatives are partial antagonists or agonists of

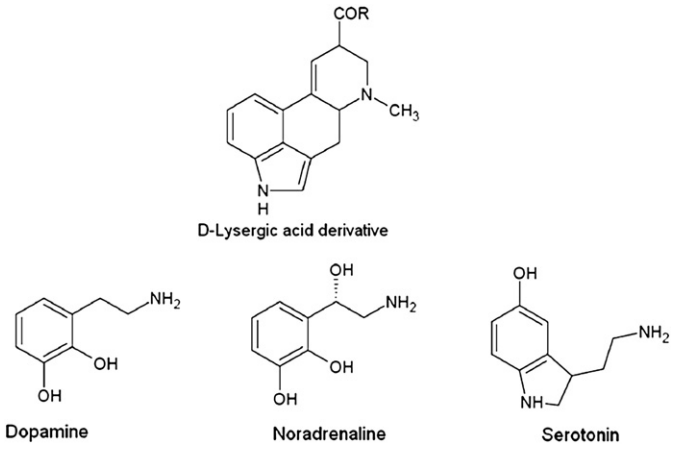

Fig. 3. Structural similarities between the derivatives of D-lysergic acid and neurotransmitters dopamine, noradrenalin and serotonin.

these receptors, which may be the cause of their cardiovascular side effects. The effect of ergot alkaloids on α_1 receptors is described in Görnemann et al. (2008). Agroclavine was also mentioned to show antibacterial effects (Schwarz and Eich, 1983) and cytostatic effects in LSI78Y mouse lymphoma cells (Glatt et al., 1987), but these finding are not supported by more recent studies.

3. Production of ergot alkaloids for pharmaceutical industry

3.1. The fungus Claviceps

There are 36 members of genus *Claviceps*, which can infect about 600 species of monocotyledonous plants. The *Claviceps* genus includes some economically important species because they infect agronomically valuable plants (Bové, 1970). A prominent member, *C. purpurea*, is spread worldwide and has the widest host range of any *Claviceps* species. It can infect about 400 species of grasses (Taber, 1985), but its most typical hosts are rye, wheat and barley (Loveless, 1971). *C. africana*, *Claviceps sorghi* and *Claviceps sorghicola* infect *S. bicolor* (Haarmann et al., 2009) grown in eastern and southern Africa, Southeast Asia, Japan, South America and Australia, *Claviceps gigantea* is found on *Zea mays* in central Mexico, *Claviceps paspali* on *Paspalum* species and *C. fusiformis* on *Pennisetum americanum* (Pažoutová and Parbery, 1999). In all *Claviceps* species, the infection is strictly targeted to the host ovaries (Parbery, 1996).

C. purpurea exhibits a life cycle shown in Fig. 4, which is typical for the *Claviceps* species. The sexual life cycle starts with an infection of unfertilized rye ovaries (Fig. 5A). Specialized penetration structures are not known in *C. purpurea*, indicating that the cell wall is passed due to enzyme secretion (Haarmann et al., 2009). Conidia are transported to the plant by wind or insects, the plant cuticle is penetrated and fungal hyphae colonize ovarian tissues. After 5 to 7 days post infection, the first macroscopically visible sign of infection, production of honeydew, can be detected (Fig. 5B). This liquid contains plenty of sugars attractive for insects and is filled with conidia, which enables secondary infection (Swan and Mantle, 1991; Tenberge, 1999; Tulasne, 1853). *C. purpurea* honeydew also contains inhibitors of conidial germination of many other fungi (Cunfer, 1976). The honeydew production stops after about 2 weeks being followed by the development of a rigid stage sclerotium, composed of a compact mass of hardened fungal mycelium (Tenberge, 1999; Tudzynski and Scheffer, 2004) as shown in Fig. 5C. After 5 weeks post infection, sclerotia mature (Kirchhoff, 1929). Sclerotia can survive unfavorable conditions in winter and germinate after a temperature increase in the spring (optimally at about 20 °C) on or beneath the soil surface (Kirchhoff, 1929), forming stromata composed of stalks with spherical capitula that grow in a phototropic manner to reach the air

Fig. 4. Life cycle of the fungus *Claviceps purpurea*: 1) opened rye floret, 2) hyphae invasion of the rye ovary, 3) honeydew production, 4) reinfection by an insect, 5) mature sclerotia formation, 6) overwintering of sclerotia, 7) germinating sclerotia, 8) release of ascospores.

(Hadley, 1968). In nature, sclerotia are the unique structures, which produce all types of ergot alkaloids (Ramstad and Gjerstad, 1955).

3.2. Field production on rye

The fungus *C. purpurea* is a species widely used in the pharmaceutical industry for its ability to produce ergot alkaloids. Clavine alkaloids, being derivatives of tetracyclic ergoline ring structure, are produced by various types of fungi. On the other hand, ergopeptines, peptidyl derivatives of D-lysergic acid amides, are produced only by some members of *Clavicipitaceae* family and their preparation by organic synthesis is economically unprofitable. The world production of ergot alkaloids reaches thousands of kilograms annually that include both ergopeptines and semisynthetic ergot alkaloid derivatives, e.g. cabergoline and pergolide (Cvak, 1999). In 2010, the total world production was about 20,000 kg, into which the field cultivation contributed about 50% (Vít Kubesa, Teva Czech Industries, Opava, Czech Republic; personal communication).

The majority of characterized strains of *C. purpurea* and other *Claviceps* species are able to synthesize ergot alkaloids only during the parasitic part of their life. The infection of the plant host by fungal spores initiates the sexual part of the *Claviceps* life cycle. Emerging hyphae invade ovary in the florets and then start to produce masses of new spores and sugar-rich exudates. Approximately 2 weeks later, affected ovaries are transformed into sclerotia where plectenchyma fungal cells initiate the ergot alkaloid biosynthesis (Tudzynski and Scheffer, 2004). Long-term breeding by random mutagenesis and the selection of highly-producing strains of *C. purpurea* allowed an increase in the ergot yield from 400 kg per hectare in the 1940s to over 1 ton per hectare nowadays in addition to increasing the alkaloid content as much as 1.5% of the sclerotia dry mass.

The traditional method of ergot production is based on spraying a conidial suspension onto a field-cultivated rye (*Secale cereale*). From the 1990s, hybrid rye lines with induced male-sterility, such as *Hyclaro* (Rentschler Biotechnologie, Laupheim, Germany), were introduced as the host because their unpollinated florets stay open longer and thus extend the period of susceptibility to ergot infection, which leads to a significantly better yield of generated sclerotia (Németh, 1999). Moreover, under natural conditions, *C. purpurea* usually attacks unfertilized ovaries and the process of infection obviously mimics the course of pollination (Tudzynski and Scheffer, 2004). An important benefit of the field ergot production is the wide variability of strains available for the production of specific types of alkaloids and their better genetic stability compared to the mutant strains used for submerged cultures. The yield of field production can reach 1–2 tons of sclerotia and from 10 kg (Tudzynski et al., 2001) to 20 kg (TEVA Czech Industries, in present) of ergot alkaloids per hectare. However, the field production can be dramatically influenced by climatic conditions of the particular year as well as by the quality and uniformity of used hybrid rye. Hence, unfavorable combinations of these factors may even result in a seasonal failure of the production (Vít Kubesa, personal communication).

3.3. Production in fermentation plants

Regarding the industrial production of ergot, there are reports on the usage of stationary surface cultivations and submerged cultures, the latter being nowadays a dominant method how to grow the *Claviceps* fungus independently of its host. A stationary cultivation using plastic bags was developed in the 1970s and used for both alkaloid production (Kybal and Vlček, 1976) and preparation of inoculums for field cultivation (Malinka, 1999).

Only some selected mutant strains have the ability to produce alkaloids in submerged cultures; the production strategies were extensively reviewed by Malinka (1999). Cells with this ability, which are called sclerotia-like cells, are shorter and thicker than the normal ones with a robust cell wall and large vacuoles (Spalla, 1973). However, a degeneration process that leads to a loss of the sclerotia-like cell

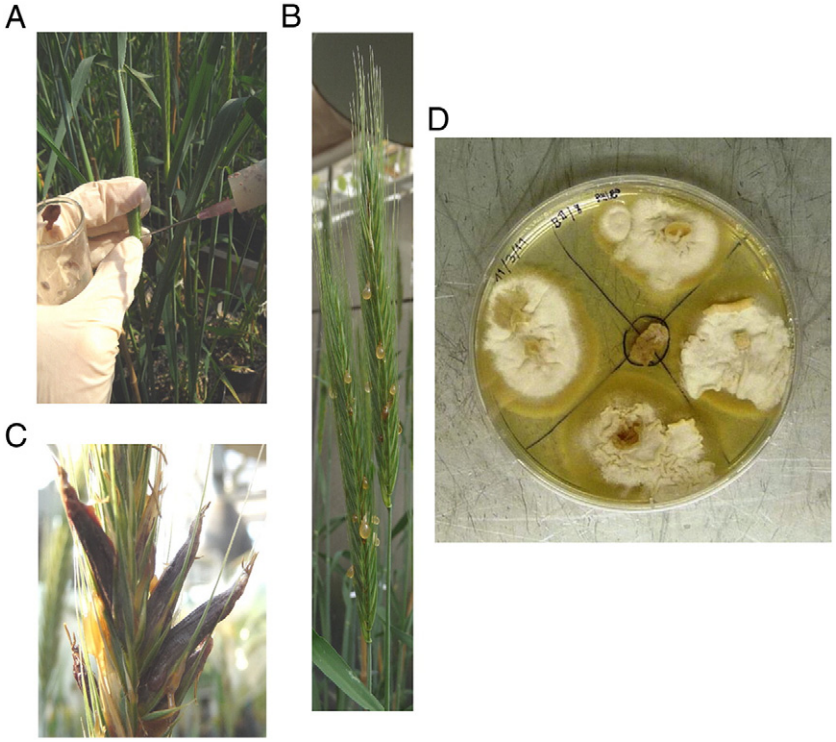

Fig. 5. Various stages of the growth of *Claviceps purpurea*: A) inoculation of rye with conidia suspension, B) honeydew production on rye, C) matured sclerotia growing on rye, D) selection of *Claviceps purpurea* transformants on a Petri dish: protoplasts of the industrial strain GAL404 were transformed with a linearized plasmid bearing *ble* gene conferring phleomycin resistance; a selective growth of transformed mycelia (on the plate periphery) contrary to untransformed mycelia (in the center) on BII agar plate with 100 μg/ml phleomycin.

morphology and lowered alkaloid production is relatively frequent; therefore a continuous selection is needed to maintain a good production strain (Malinka, 1999). Fermentation is nowadays used mostly to produce paspalic acid, which isomerizes to D-lysergic acid, and some derivatives of paspalic acid that serve as a starting material for preparation of semisynthetic alkaloid derivatives. D-lysergic acid can be also obtained by alkaline hydrolysis of simple amides, ergometrine and even ergopeptines (Rucman, 1976).

Production of ergopeptines by fermentation is much more complicated and requires specific conditions. Cultivation media induce the formation of sclerotial-cell like morphology by two mechanisms, substrate limitation and favoring oxidative metabolism. Alkaloid production requires a non-inhibitory, slowly metabolized carbon source such as mannitol, sorbitol or sucrose at a high concentration (sucrose 300 g/l). Moreover, a high osmotic pressure (10–20 bar) of the medium is prerequisite for the formation of sclerotia-like cells while inhibiting conidiation (Kobel and Sanglier, 1986). *Claviceps* converts sucrose, which is the main sugar in plant phloem sap and serves as a natural nutrient of the fungus, to fructofuranosyl-oligosaccharides. These oligosaccharides are used as an energy source in the late stage of the fermentation, which is the key period for the production of ergopeptines. Production media usually also contain an organic acid of the tricarboxylic acid cycle as a carbon source, which under low phosphate promotes the high level of oxidative metabolism in the *Claviceps* cells that is necessary for the biosynthesis of secondary metabolites.

Biosynthesis of ergot alkaloids in axenic cultures is positively regulated by tryptophan, which acts as a precursor and inducer, and negatively by phosphate and ammonium, which repress biosynthesis (Sočič and Gaberc-Porekar, 1992). Typically, alkaloid synthesis starts after depletion of phosphate from the medium, whereas an excess of phosphate leads to undesired growth-linked repression of alkaloid production. General reviews about ergot alkaloid production in submerged cultures have been published by many authors including Esser and Düvell (1984); Robbers (1984); Křen et al. (1994); Sočič and Gaberc-Porekar (1992), and Flieger et al. (2004).

3.4. Genetic transformation of Claviceps

The possibility of genetic transformation of *Claviceps* brought a new tool, not only for obtaining useful knowledge about the genetic principles of ergot alkaloid formation and attractive parasitic lifestyle of the fungus by novel studies of knock-out mutants, but also for having an effect on the quality and/or quantity of ergot alkaloid production for industrial purposes.

Undoubtedly, the most popular method for the genetic transformation of *Claviceps* is protoplast transformation in a medium of high osmotic pressure. A successful method based on protoplast preparation using lytic enzymes was first described in 1989 (Engelenburg et al., 1989) and was later improved (Mey et al., 2002). There are many commercially available enzymes or their mixtures suitable for *C. purpurea* protoplast preparation, such as Lysing enzyme and Driselase (Mey et al., 2002) or β-glucuronidase (Keller et al., 1980). Ca^{2+} ions are universal component of the transformation mixture, while the high osmotic pressure can be adjusted by adding PEG (Engelenburg et al., 1989; Mey et al., 2002). Transformants can then be selected based on acquired antibiotic resistance (Engelenburg et al., 1989; Mey et al., 2002) or by using pyrimidine auxotrophy (Smit and Tudzynski, 1992). Hygromycin (1.0 mg/ml; Comino et al., 1989) and phleomycin or bleomycin (0.1 mg/ml; Mey et al., 2002) are the most often used selection antibiotics. The genes responsible for acquired resistance have been described as *Hph*, encoding hygromycin B phosphotransferase, and *ShBle* gene, coding for a protein binding to bleomycin and inhibiting its DNA cleavage activity (Dumas et al., 1994). Selective growth of transformed vs. untransformed mycelia on a medium containing phleomycin is shown in Fig. 5D (Hulvová et al., 2009).

Technical procedures for *C. purpurea* transformation with the usage of both homologous and non-homologous recombination have been developed (Comino et al., 1989; Smit and Tudzynski, 1992). For the expression of transgenes, constitutive fungal promoters, such as *trpC* (Engelenburg et al., 1989) and *gpdA* from *Aspergillus nidulans* (Lorenz et al., 2010; Scheffer et al., 2005), are used.

Transformation of *C. purpurea* by *Agrobacterium tumefaciens* has been attempted, but no successful procedure has been described yet, although many procedures of *Agrobacterium*-mediated transformation of filamentous fungi are already known (Groot et al., 1998; Meyer et al., 2003; Michielse et al., 2005; Mullins et al., 2001). Because of the advantages of this method, such as one copy insertion or stable transformant preparation, development of the procedure for *Claviceps* transformation would be very useful.

4. Biotechnological prospectives of production improvement

Improvement of biotechnological processes relies on various transformation approaches used during the last several decades. Up to now, only randomly mutated strains of *C. purpurea* have been exploited in the industrial production of ergot-stock. A recently sequenced EAS cluster of fourteen genes sheds more light on the metabolic pathways and regulations of ergot alkaloid production. Thus, a directed overexpression of certain genes or targeted up-regulation of the whole cluster could significantly increase the yield of ergot alkaloids.

Production amounts and a qualitative composition of synthesized alkaloids vary significantly among different isolates of the species *C. purpurea* (Pažoutová et al., 2000). Long terminal repeat sequences and nonautonomous transposons were detected within the ergot alkaloid cluster indicating that spontaneous rearrangements can occur very frequently. Moreover, the cluster for ergot alkaloid production, in *A. fumigatus*, was found to lie in the telomeric region, which is often subject to recombinations, chromosomal breaks and duplications (Coyle and Panaccione, 2005). A recent evolutionary study revealed that the *dmaW* gene passed through many duplications and losses (Liu et al., 2009). The genes of ergot alkaloid cluster are probably also frequently subjected to loss of function mutations, deletions etc. Such an event can be demonstrated on a pseudogene of *dmaW* that was found in an unspecified strain of *C. purpurea* (GenBank AJ312752).

4.1. Manipulation of EAS cluster of Claviceps

Genes encoding all the enzymes participating in ergot alkaloid biosynthesis are likely to be located within the EAS cluster (Fig. 2B), but not all the genes involved in the pathway have been identified and several of detected ORFs still have putative or unknown functions. Recently, an evolutionary study of the alkaloid gene cluster has been accomplished including *Claviceps* species that produce different spectra of alkaloids (Lorenz et al., 2007). *A. fumigatus* cluster, whose end-products are clavine alkaloids, contains all ORFs present in the EAS cluster of *C. purpurea* except for the three genes coding for non-ribosomal peptide synthases, *cloA* gene and a functional form of *easH*. Moreover, frameshifts and partial truncations were found in two ORFs for monooxygenases that are responsible for the final production of D-lysergic acid from a chanoclavine precursor. The comparison of *Claviceps hirtella* and *C. fusiformis* is particularly interesting. Despite their very close relationship, the former fungus can synthesize lysergic acid derivatives, while the latter one lacks this ability. The *C. hirtella* cluster encodes an additional functional monomodular non-ribosomal peptide synthase and two monooxygenases, allowing the production of ergometrine, a single amino acid derivative of D-lysergic acid (Lorenz et al., 2009). In general, the overall organization of the cluster is highly conserved; however rearrangements and point mutations dramatically change the expression and functionality of the enzymes involved in the pathway and consequently both quality and

quantity of produced alkaloids. A detailed characterization of alkaloid gene clusters in other members of the genus *Claviceps* is needed as for instance main products of *C. africana* and *C. gigantea* are unique dihydroclavine derived alkaloids. Hence, a combination of different production strains can improve the quality of produced ergot-stock in biotechnological applications.

The gene *dmaW* is common to all *Claviceps* species described to date; its product catalyzes the first specific step in ergot alkaloid biosynthesis and has the key regulatory function in the pathway (Wang et al., 2004). Feeding experiments with tryptophan led to increased activity of DMAT synthase, which resulted in increased total amount of produced alkaloids (Krupinski et al., 1976). Thus, one can expect that ubiquitous overproduction of DMAT synthase enzyme can result in a higher accumulation of dimethylallyltryptophan and reinforcement of further metabolic steps. The actual concentrations of L-tryptophan and dimethylallylpyrophosphate do not seem to be a limiting factor as they are products of the primary metabolism.

The *EasC* gene, with a high homology to catalases, is another gene of interest in the ergot alkaloid cluster. A knock-out mutant of *C. purpurea* did not produce any alkaloids and the transcripts of other cluster genes were down-regulated (Haarmann and Tudzynski, 2006). An elimination of the *easC* gene in *A. fumigatus* led to the same effects and an exogenous addition of chanoclavine to the growth medium restored the alkaloid production. Hence, the *easC* gene product plays an essential role in the metabolic pathway upstream of chanoclavine (Goetz et al., 2011). Hypothetical catalase easC can either consume hydrogen peroxide generated by chanoclavine synthase or function as a general regulation factor of the whole metabolic cascade (Goetz, 2008).

Unlike other secondary metabolic clusters in fungi, no transcription factor gene has been identified among the ergot alkaloid cluster ORFs yet. Originally, the protein encoded by the *easG* gene was thought to be a good candidate since it shows a homology to Nmr proteins that are involved in regulation of nitrogen assimilation (Tomsett et al., 1981). However, easG was very recently found to catalyze the final step of chanoclavine-I aldehyde conversion to agroclavine (Matuschek et al., 2011).

Based on the published finding, overexpression of either *easC* or *easG* gene was predicted to promote ergot alkaloid biosynthesis in *C. purpurea*. Recently, we used a protoplast method to prepare *C. purpurea* mutants overexpressing genes *easC* and *easG* under a constitutive promoter of glycerol-3-phosphate dehydrogenase (GAPDH) from *A. nidulans* (Hulvová et al., 2010). Confirmed transformants of each gene were applied on rye growing in a greenhouse, but chemical analysis showed no significant increase in the production of ergot alkaloids. However, the native expression of the two genes in the wild type *C. purpurea* was already very strong and reached the level of GAPDH promoter-driven overexpression 20 days after infection (Hulvová et al., unpublished results).

Genetic engineering of the proteins involved in related signaling cascades appears to be another promising way on how to influence ergot alkaloid biosynthesis. Since ergot alkaloid production is dependent on a high osmotic pressure in the culture medium or under natural condition on the production of honeydew, one can expect that receptors and transmitters active in osmoregulation can be good candidates. Pioneering research at this area has been already initiated (Lorenz et al., 2009; Mey et al., 2002).

In fungi, secondary metabolite clusters are often located in a heterochromatin, near the telomere region (Galagan et al., 2005; Nierman et al., 2005; Rehmeyer et al., 2006). Thus, it is speculated, that the EAS cluster of *C. purpurea* may be also located in the subtelomeric area (Lorenz et al., 2007). In *Aspergillus* spp., expressions of many biosynthetic gene clusters are regulated by histone methyltransferase LaeA. An overexpression of the *laeA* gene in *A. nidulans* led to an increased expression of the cluster genes and product formation (Bok and Keller, 2004). The overexpression of *laeA* gene from *A. nidulans* in the *C. purpurea* P1 strain did not lead to increased expression of EAS cluster genes or ergot alkaloid production (Lorenz et al., 2009). Nevertheless other genes coding for the enzymes participating in methylation and acetylation/deacetylation of histones can be also good candidates for improvement of EAS cluster expression. Recently, a formation of heterotrimeric complex of LaeA with two other proteins, VelB and VeA, was shown to be essential for its activity in nucleus to control secondary metabolism gene transcription. Deletion of both genes led to defects in fungal sexual reproduction and ceased production of some secondary metabolites (Bayram et al., 2008). Furthermore, expression of VeA is down-regulated and its accumulation in nucleus ceases after illumination (Stinnett et al., 2007). On the other hand, VelB protein lacks any nuclear targeting signal and its transfer to nucleus depends solely on VeA. Thus, an overexpression of VeA under a constitutive (light independent) promoter and/or expression of VelB with a specific nucleus targeting signal could lead to increased production of ergot alkaloids under natural light conditions (Bayram et al., 2008).

4.2. Improvement of rye hybrid lines with male-sterility

Today, hybrid rye lines with induced cytoplasmic male sterility are widely used in different breeding programs and for heterosis seed production. Fertility can be restored by a suitable parent line bearing restorer gene(s) (Geiger and Miedaner, 2009). Commercial hybrid rye lines with cytoplasmic male-sterility are currently the only suitable host for ergot stock field production. The most crucial factor biasing the quantity of annual ergot yield, besides the weather conditions which influence the progression of the infection in flowering rye plants, is the purity of the rye line. To keep the cytoplasmic male sterility line, regular crossing with the restorer line has to be performed with vigorous selection of male-sterile seed stock. This procedure is very agronomically demanding and significantly elevates production costs. Even though, maintenance of high-quality seed stock offering 100% male-sterile plants for ergot production is almost impossible because rye is an effective wind pollinator and transfer of pollen containing restorer gametes to long distances can occur on either ergot production field or hybrid seed production field. To avoid losses in ergot production by unexpected leakage of pollen, fields for production of hybrid seed and ergot stock should be located out of traditional rye-growing areas.

Ectopic expression of the barnase gene, ribonuclease from *Bacillus amyloliquefaciens*, in tapetum cells of developing anthers leads to destruction of pollen and therefore to male-sterile plants. A system utilizing the barnase gene and the fertility-restorer gene, barstar, has been introduced to many crops and is one of the most widely used transgenic approaches in modern agriculture (Williams, 1995). Due to the fact that rye is one of the most recalcitrant plant species for tissue culture and genetic transformation (Popelka and Altpeter, 2003), and low economic impact of rye cultivation in global agriculture, transgenic rye lines bearing barnase–barstar system have not yet been prepared. Thus, introduction of the barnase gene into the rye genome together with the linked selection gene (e.g. herbicide resistant) can produce an easy, selective and inexpensive system for obtaining a pure male-sterile rye line suitable for ergot production in the future. Seed stock of the sterile line can be maintained by pollination crossing with wild type plants. The barnase gene, as well as a selective marker, act as dominant alleles in the transgenic genome and thus a 100% sterile population of rye plants can be selected every year by proper application of herbicide.

4.3. Susceptibility of rye ovaries to the infection by different Claviceps strains, pathogenicity markers

Susceptibility of rye plants to the infection by ergot is another key determinant of a good yield in the field production of ergot alkaloids. The infection depends on many agronomical parameters, but primarily on the fertility of the host cultivar, its pollen shedding and

Table 1
Virulence and pathogenicity-related proteins of *Claviceps*.

Gene	Protein	Function	Reference
Pg1/2	Polygalacturonase	Pectin degradation in rye ovary	Oeser et al. (2002)
Rac	GTPase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Xyl2	Endo-1,4-β-xylanase	Hydrolysis of xylans	Tudzynski and Scheffer (2004)
Cdc42	GTPase	Sensing of polarity and sporulation	Scheffer et al. (2005a)
Cla4	P21-activated kinase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Cot1	Ser/Thr kinase	Sensing of polarity and branching	Scheffer et al. (2005b)
Mid1	Ca^{2+} channel	Signaling Ca^{2+} uptake after membrane distension	Bormann and Tudzynski (2009)
Mk1/2	MAP kinase	Pathogenicity related signaling	Mey et al. (2002a) Mey et al. (2002b)
Tf1	CREB-like TF	Oxidative stress responses	Nathues et al. (2004)
Hk2	His kinase	Signaling of oxidative stress and osmosensing	Nathues et al. (2007)
Nox1	NADPH-oxidase	ROS generation	Giesbert et al. (2008)

duration of flower opening. For direct testing of the level of pathogen virulence, male-sterile hybrids grown in the greenhouse conditions are used. Several pathogen knock-out transformants were prepared to study the molecular aspects of virulence and host–pathogen interaction. In general, virulence associated genes can be divided into three main categories, those encoding the enzymes participating in degradation of host cell wall; enzymes scavenging reactive oxygen species that are produced by the host as a pathogen protection response; and various regulatory proteins involved in signaling pathways or functioning as transcription factors. *Claviceps* proteins known to be important for fungal virulence and pathogenicity are listed in Table 1.

As the fungus mimics pollen tube growth during the infection, only mild or no host defense reaction is usually observed (Tudzynski and Scheffer, 2004). On the other hand, recent high-throughput screening of EST clone libraries from infected rye tissues revealed expression of various proteins related to general pathogen defense responses (Oeser et al., 2009). Nevertheless, actual pathogen-induced expression of these genes was not shown and none of them was yet confirmed to be involved in the resistance to the pathogen infection. Since the whole genome sequencing of *C. purpurea* strain P1 has been recently accomplished and a rough draft will soon be publically available (Paul Tudzynski, personal communication), a future transcriptomic study shall reveal presumptive host resistance related genes or other genes specifically switched during the ovary invasion as potential easy-to-detect markers of the pathogenicity process. Genome sequencing of other *Claviceps* species is in progress worldwide, among them *C. africana* is being sequenced using a high-throughput shotgun technology in our laboratory.

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Supplement 7.2

Research article

Improving field production of ergot alkaloids by application of gametocide on rye host plants

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Improving field production of ergot alkaloids by application of gametocide on rye host plants

Research Paper

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Ergot alkaloids are widely used in the pharmaceutical industry in drug preparations for treating migraines and Parkinson's disease, inducing uterine contraction, and other purposes. Phytopathogenic fungi of the genus *Claviceps* (e.g. *C. purpurea*) comprise a major biological source of ergot alkaloids. Worldwide industrial production of these alkaloids derives almost equally from two biotechnological procedures: submerged culture of the fungus in fermenters and field parasitic production in dormant fungal organs known as sclerotia (also termed ergot). Ergot yields from field cultivation are greatly affected by weather and also can be much reduced by pollen contamination from imperfectly male-sterile rye, as only unfertilized ovaries can be infected by *C. purpurea* spores. Two substances with gametocidal effect – maleic hydrazide and 2-chloroethylphosphonic acid – were tested during three consecutive seasons in small field experiments for the ability to induce or amplify the male sterility of rye as well as the impacts on germination of *C. purpurea* spores and general vitality of rye host plants. Maleic hydrazide was proven to be a highly effective gametocide on both a fertile rye variety and a variety with imperfectly induced cytoplasmic male sterility. It showed negligible effect on germination of *C. purpurea* spores. Both accurate dosaging of the active gametocidal compound and timing of the application just 2–3 weeks before onset of anthesis proved crucial to achieving high ergot yield with minimum grain impurities.

Introduction

The biotrophic fungus *Claviceps purpurea* is a member of the globally distributed *Clavicipitaceae* family of plant pathogens and epiphytes. Although the fungus can infect more than 70 grass species, the most susceptible host is rye, *Secale cereale* [1]. Infection begins with adhesion of an ascospore transmitted by wind or an insect vector to the stigma of host florets, where it competes with pollen grains. If the spore germinates through the pistil, hyphae start to colonize the ovary and produce conidiospores which spread a secondary infection to surrounding florets and plants. Flower structures are later transformed into rigid sclerotia, where ergot alkaloids (EAs) begin accumulating approximately 3 weeks after the primary infection [2].

Belonging to the class of indole derivatives, EAs are widely used in the pharmaceutical industry for their ability to act as agonists of serotonin, dopamine and adrenergic receptors [3]. The genome of *C. purpurea* contains a 68.5 kb cluster of 14 mainly unique genes, the activation of which leads to EA production [4]. EAs can be

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divided into three major groups: clavine alkaloids; D-lysergic acid and its simple derivatives produced also by other species; and *C. purpurea* exclusive ergopeptines, which are tripeptide derivatives of D-lysergic acid amide. Because organic synthesis of ergopeptines is very difficult and not at all profitable, their production by fungal cells with subsequent extraction and purification is the preferred method of preparation used by pharmaceutical companies. Most *Claviceps* species and strains are able to produce EAs only during their parasitic lives, and that is especially true of those producing ergopeptines as a final product. Industrial strains of *C. purpurea* producing sclerotia containing large amounts of ergopeptines have been obtained through long-term breeding and random mutagenesis. These strains can accumulate as much as 2% of EA in the dry weight of sclerotia. Ergot yields become economically profitable at around one-half ton per hectare of infected rye field [5,6] (Teva Czech Industries, recent years; unpublished results).

Parasitic production in the field is based on spraying or injection of non-pollinated rye plants in the earing phase with a conidial suspension of *C. purpurea*. During the 1980s, introduction of hybrid rye lines with cytoplasmic male sterility, such as the cultivar Hyclaro (Rentschler Biotechnologie, Laupheim, Germany) contributed to significantly increased ergot yield [6]. These hybrid lines produce no pollen; their florets stay open for a longer period and are thus more susceptible to the infection. Nevertheless, there exist many additional factors which can negatively affect field production. In addition to climatic conditions, the quality and homogeneity of hybrid seed stock are crucial. Even a tiny amount of contamination with the parental component during hybrid seed stock preparation can produce a sufficient amount of pollen on the production field to cause undesirable formation of grains. Sclerotia formation is then reduced, and that in turn significantly increases the cost of drug preparation. Hybrid sterile lines are maintained by crossing fertile parental components. Inasmuch as one of the lines always carries the genes restoring fertility, individuals of this line must be precisely eliminated during hybrid seed propagation. Cross-contamination with parental components can occur during plant propagation in extreme conditions, where penetration of the genes inducing male sterility is not absolute [7]. Genetic purity failure due to improper agro-technical practices results in the presence of fertile individuals greater than the acceptable threshold of 0.4% in the sterile hybrid population. Such a seed stock with reduced male sterility is then not suitable for commercial ergot production in the field.

For cereals and other crops, male sterility can be also induced chemically. A gametocide is a morphogenetic poison that is typically used to treat the plants in the stage of generative organ initiation [8]. It is able to inhibit the creation of microspores in anthers or degrade sporoderm in pollen grains and thus prevent their germination. Depending on dosage, gametocides can cause partial or full male sterility. Determining the appropriate dose is very important, as higher doses can also negatively affect plant growth and the development of female reproductive organs. It is assumed that gametocides induce either a hypersensitive reaction or a phytohormonal imbalance, thereby distorting the development of male gametophyte tissues [9]. Hypersensitive reaction can be induced by substances based upon organophosphates or halogen derivatives of carboxylic acids, while phytohormonal imbalance can be induced by antagonists or agonists of phytohormones, such as 2-chloroethylphosphonic acid, gibberellic acid, and maleic hydrazide. In 1949, Schoene and Hoffman [10] first described the inhibitory effect of maleic hydrazide (MH; 1,2-dihydro-3,6-pyridazinedione) on plant growth. Later, the compound was successfully used as a male gametocide for many plant species, including coriander [11], rice [12], and wheat [13]. MH is an auxin and gibberellic acid agonist [14] that is commonly used as a diethanolamine salt or sodium salt soluble in water. MH inhibits the division and extension growth of certain cell types and prolongs the period of dormancy in many plant species [15].

Ethrel (ETH; 2-chloroethylphosphonic acid, also known as ethephon), which upon its being metabolized by the plant is converted into phytohormone ethylene [16], is the most widely used growth regulator for inducing ripening and enlarging fruit size. Major effects of ethylene include to induce fruit ripening, inhibit extension growth, facilitate the onset of senescence, and, in some species, also to inhibit flowering [17]. ETH has been studied for gametocidal effect in many plant species. It is used as a convenient gametocide for wheat, wherein it adequately induces male sterility while maintaining female fertility when applied at an appropriate pre-meiosis growth stage [18]. When it was applied to pearl millet, ETH also showed great ability to induce male sterility without negatively affecting female fertility. On the other hand, the susceptibility of ETH-treated pearl millet plants to infection by the parasitic fungus *Claviceps fusiformis* did not change in comparison to that of untreated control plants [19].

The aim of the present work was to study the effectiveness of gametocides on the parasitic production of ergot in a series of field experiments. The results obtained show that appropriate application of a gametocide greatly eliminates the ability of pollen to germinate and increases the purity of ergot harvests while maintaining high yields for both a rye hybrid with male sterility as well as classical fertile varieties which previously were assumed to be unsuitable for effective industrial production of EAs.

Materials and methods

Materials

Winter rye cultivar L25P × L130N with cytoplasmic male sterility was developed by the Plant Breeding and Acclimatization Institute (Radzikow, Poland), and hybrid seeds were propagated from parental components by local farmers. Winter rye population variety Selgo was obtained from Selgen (Stupice, Czech Republic). The *C. purpurea* [Fr.] Tul. strain CCM 8405 mutated by gamma rays and bred for high EA content is owned by Teva Czech Industries [20].

Rye field preparation

The experimental field is located in Olomouc – Holice (Czech Republic; 49°34′30″ N–17°17′07″ E). Seeds were sown in the 40th week of the year at a seeding rate of 180 kg per ha into brown-earth soil on small experimental plots, each with an area of 10 m^2. Plants were treated with a herbicide and an insecticide in the same year and again 1 or 2 weeks before the first gametocide application. A mineral fertilizer containing 27% nitrogen was conventionally applied twice during springtime. A field with the fertile rye variety (16 × 2 plots) was approximately 100 m distant from an identically sized field of the sterile variety, both of which were surrounded by border strips of wheat. Plots were treated with Fazor® (Crompton Manufacturing Company; 600 g/kg of MH) or Cerone® (Bayer

CropScience; 480 g/L of ETH) at 1-week intervals using a SOLO 485 backpack sprayer in a randomized pattern, each dosage and application time was in two to four replicates. Preparations were dissolved or diluted in tap water and supplemented with the surfactant Rollwet™ (Agrovista, UK) in the final concentration 0.1% (v/v).

Conidiospore preparation and field infection

No sooner than 5 days following the final gametocide application, ears at phenological stage BBCH 63 (for the Selgo variety) and BBCH 61 (for the L25P × L130N variety; [21]) were inoculated using conidia suspension of the *C. purpurea* strain CCM 8405. Inoculation was performed by piercing ears with a grid of metal needles regularly dipped into the conidial suspension. The conidial suspension was prepared by homogenizing 4 g of dried mycelia [22] using a disperser in 1 L of 0.1% corn starch solution buffered with 0.1 M K^+-phosphate buffer to pH 5.3.

Harvest and yield evaluation

The rye was harvested in the 28th week of the year at phenological stage BBCH 89 using a Hege 160 plot combine. The mixture of grains and sclerotia obtained from each plot was dried separately for 1 month at temperature 30–35°C and then weighed. An amount up to 100 g was manually categorized into grains, small sclerotia (longitudinal size up to 1 cm), and primary sclerotia (larger than 1 cm). Three randomly collected aliquots of 50 g were categorized from a plot upon which yield exceeded 100 g.

Spore inhibition assay

The quantity 0.5 g of mycelium grown on solid sporulation Mantle agar [23] was added to 250 mL of 150 mM NaCl and homogenized using a disperser at room temperature. The suspension was diluted to concentration 5×10^{10} conidia per mL. Fazor® was added to a final MH concentration corresponding to a series from 2.5 to 10 g/mL of Mantle agar, upon which it was spread in 0.5 mL aliquots. Plates with identical MH concentrations were grown at 23°C in two replicates. The quantity and morphology of germinating conidia were microscopically evaluated after 24 or 48 hours, respectively.

Ergot alkaloids quantification

Ergot free from any impurities was ground in a ball mill to particle size up to 0.3 mm, and 0.5 g of the product was dissolved in 50 mL of extraction buffer (26% ammonium hydroxide and 90% acetone, 1:100, v/v) on a shaker for 2 hours. A 10 mL aliquot was filtered through glass wool and dried using a vacuum evaporator at 50°C. The residue was dissolved in 2 mL of 90% methanol, filtered through a 0.22 μm nylon filter and analyzed for EA content using ultra high performance liquid chromatography on a Nexera system (Shimadzu) equipped with a C18 reverse-phase column (Zorbax RRHD Eclipse Plus, 1.8 μm, 2.1 mm ID × 50 mm, Agilent). An EA reference mixture was prepared in 90% methanol and contained 0.001% (w/v) of each of the following alkaloids: ergotamine, ergostine, 8-hydroxyergotamine, ergocornine, α-ergokryptine, β-ergokryptine, ergocristine, ergogaline, ergotaminine, ergostinine, ergocorninine, α-ergokryptinine, β-ergokryptinine, ergogalinine, and ergocristinine (all from Teva Czech Industries). The analytes were eluted with solvents A (36 mM triethylamine/phosphate, pH 4.4, and acetonitrile, 4:1, v/v) and B (H_2O and acetonitrile, 1:4, v/v) using the following gradient: 0 min, 9% B; 10–12 min, 9–13% B; 12–14 min, 13–29% B; 14–16 min, 29–44% B; 18–20 min, 44–59% B; 26–28 min, 59–9% B, at flow rate 0.4 mL/min and column temperature of 30°C. Monitoring was at 317 nm.

Results and discussion

Selection of gametocide with positive effect on ergot yield

Plant protection products commercially available and certified for use in the European Union were surveyed for the presence of active components having gametocidal effect. Finally, two products containing ETH and MH as active compounds, respectively, were selected. These were Cerone®, which is used to increase cereal resistance to lodging, and Fazor®, which is used as a sprouting suppressor for potato tubers or onion bulbs. In the 2012 season,

TABLE 1

Effects of different dosages of MH and ETH on grain production and ergot yield on hybrid rye L25P × L130N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2012 season)

Dosage (kg/ha) applied at BBCH 51	Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
None	82 ± 2	2750 ± 308	42.9	57.1	1395 ± 106	100.0
Maleic hydrazide						
2	69 ± 1	26 ± 6	22.9	77.1	484 ± 32	0.9
4	69 ± 2	6 ± 2	42.6	57.4	193 ± 21	0.2
8	69 ± 1	3 ± 0	44.4	55.6	127 ± 100	0.1
15	59 ± 1	None	None	None	None	0.0
Ethrel						
2.5	64 ± 2	1730 ± 252	80.7	19.3	674 ± 48	62.9
5	60 ± 1	1994 ± 704	75.5	24.5	824 ± 93	72.5
10	61 ± 1	102 ± 16	66.2	33.8	302 ± 12	3.7
20	55 ± 1	86 ± 22	38.8	61.2	294 ± 32	3.1
40	57 ± 1	202 ± 41	54.5	45.5	132 ± 29	7.3
80	52 ± 1	130 ± 40	50.9	49.1	110 ± 7	4.7
160	51 ± 1	74 ± 30	100.0	None	16 ± 4	2.7

[a] Measured at phenological stage BBCH 57.

small experimental plots with the hybrid male-sterile rye L25P × L130N were treated with doses ranging from 2 to 15 kg and 2.5 to 160 kg per ha of MH and ETH, respectively, 3 weeks before onset of flowering (BBCH 55, [21]). Higher dosages of ETH (above 20 kg per ha) showed such immediate negative effects as formation of leaf lesions, leaf rolling, and cessation of growth and ear development that were visible already a few days after the application. None of these effects were observed on plants treated with MH. Both gametocides caused reductions in plant height across the whole range of dosages used (Table 1). After treated plants as well as non-treated controls were infected by *C. purpurea* conidia, the progress of infection was followed and ergot yield estimated. The first symptoms of infection – formation of honeydew droplets and polyamine odor – were obvious 12 days after infection with the same intensity on control plots as on plots with gametocide dosages up to 4 kg and 20 kg per ha for MH and ETH, respectively. Plots with higher dosages showed significantly lower infection symptoms. The seed stock used (hybrid rye L25P × L130N) showed a high penetration of fertile individuals ranging from 2% to 20%. MH significantly decreased grain yield for treated plants already at a dosage of 2 kg per ha in contrast to non-treated plants (Table 1). ETH treatment caused a similar reduction, but only at much higher doses and while also negatively affecting plant height and vitality already at a dosage of 2.5 kg per ha. Although both gametocides also slightly reduced ergot yield even at the lowest application dosages, the decrease in contaminating rye grains in the total harvest was far more prominent, and especially so in the case of MH. In contrast to ETH, MH did not substantially affect plant vitality or growth. MH was therefore selected for further testing in subsequent seasons. ETH treatment was repeated in the 2014 season with similar results. There was a proportional reduction in plant height and grain and ergot yield with increasing ETH dosage from 1 to 5 kg per ha (data not shown), and the total ergot yield and grain/ergot ratio were much better when MH was used.

Ergot yield from fertile rye variety treated with maleic hydrazide

Although male-fertile rye varieties have not been used for industrial production in recent decades, some of them have high susceptibility to *C. purpurea* infection, and especially those with a broader range of flowering time and longer ears. It is crucial to infect fertile varieties before pollination onset as the pollen

TABLE 2

Effects of different MH dosages and application times on grain production and ergot yield for hybrid rye L25P × L130N with imperfect male sterility and fertile rye variety Selgo infected by *C. purpurea* CCM 8405 (experiment run during the 2013 season)

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Hybrid rye L25P × L130N with imperfect male sterility					
19th BBCH 47	20th BBCH 49	21st BBCH 55	22nd BBCH 59						
–	–	–	–	119 ± 6	1938 ± 370	63.7	36.3	178 ± 22	100.0
–	–	0.5	0.5	112 ± 2	844 ± 71	39.3	60.7	164 ± 114	43.6
–	0.5	–	–	113 ± 2	2245 ± 254	67.0	33.0	197 ± 18	115.8
1	1	1	1	77 ± 1	17 ± 12	66.3	33.7	74 ± 7	0.9
–	1	1	1	116 ± 1	487 ± 354	60.4	39.6	624 ± 72	25.1
–	1	1	–	113 ± 2	1317 ± 175	89.5	10.5	295 ± 19	68.0
–	1	–	–	105 ± 2	1168 ± 369	85.7	14.3	91 ± 9	60.3
–	–	1.5	1.5	108 ± 6	137 ± 48	61.5	38.5	670 ± 125	7.1
–	1.5	–	–	110 ± 2	1078 ± 87	93.5	6.5	217 ± 14	55.6
–	2	2	2	106 ± 1	90 ± 57	78.9	21.1	462 ± 49	4.6
–	2	–	–	108 ± 3	371 ± 154	83.9	16.1	193 ± 15	19.1
–	3	–	–	103 ± 3	197 ± 35	59.4	40.6	271 ± 32	10.2
MH dosage (kg/ha)				**Plant height (cm)[a]**	**Grain yield (kg/ha)**	**Small sclerotia (%)**	**Primary sclerotia (%)**	**Total ergot yield (kg/ha)**	**Grain yield (%)**
Week of the year				**Fertile rye Selgo**					
19th BBCH 49	**20th BBCH 51**	**21st BBCH 59**	**22nd BBCH 61**						
–	–	–	–	124 ± 3	4301 ± 136	61.1	38.9	190 ± 11	100.0
1	–	1	1	97 ± 3	7 ± 2	35.7	64.3	14 ± 2	0.2
–	1	1	1	118 ± 3	416 ± 113	24.7	75.3	308 ± 38	9.7
–	2	2	2	105 ± 4	114 ± 53	42.1	57.9	428 ± 58	2.7
–	3	3	3	106 ± 1	209 ± 112	27.6	72.4	485 ± 392	4.9

[a] Measured at phenological stage BBCH 69.

competes strongly with ergot conidia [6]. Since rye is cross-pollinating and the onset of flowering in the field is not synchronized, a huge amount of pollen transmitted by wind from early maturing individuals can block *C. purpurea* infection of those ovaries that were not infected early enough.

The fertile rye variety Selgo was treated with MH at different time points, beginning 3 weeks before the onset of flowering. Similarly to the case of the male-sterile variety, MH application visibly affected only plant height while not altering flower development and pollen shedding from anthers. The fertile rye was not inoculated until phenological stage BBCH 62, at which time approximately 30% of the anthers in the field were mature and produced pollen. Despite pollen production, decrease in grain formation by more than 10-fold was observed on all plots where the gametocide was applied (Tables 2 and 3). The MH concentrations used and the application during the period when male gametophytes are already in development most probably did not affect microspore formation. Rather, these led to the formation of pollen grains that were unable to germinate. The gametocide also did not affect the development of female gametophytes, as the ovaries remained susceptible to conidiospore infection and produced fully developed sclerotia (Fig. 1a). The ratio between harvested ergot and rye grain changed significantly (Fig. 1b), and ergot yield almost reached the value obtained using the hybrid male-sterile variety (Table 2). Previous studies have shown that sclerotia formed on a fertile rye are generally larger but fewer in number per ear (primary sclerotia) than are those on male-sterile rye (mostly sclerotia from secondary infection), the former condition being considered a disadvantage for ergot harvest [6]. A different proportion of primary as opposed to that of secondary sclerotia on fertile versus sterile rye treated with MH was also observed in the 2013 season experiment (Table 2). This difference was not so obvious in the following year, however, thus suggesting that the size of sclerotia might be influenced also by weather conditions.

A lower dosage of MH (1–4 kg per ha), which already significantly decreased grain formation for the fertile rye variety, showed almost no effect on ergot yield. On the other hand, the time of first

TABLE 3

Effect of different MH application times on grain production and ergot yield for fertile rye variety Selgo and hybrid rye L25P x L130 N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2014 season)

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Fertile rye Selgo					
17th BBCH 49	18th BBCH 51	19th BBCH 59	20th BBCH 61						
–	–	–	–	127 ± 6	3946 ± 957	58.6	41.4	162 ± 109	100.0
2	–	2	–	110 ± 1	38 ± 29	38.5	61.5	85 ± 61	1.0
2	2	2	–	106 ± 8	101 ± 22	54.2	45.8	69 ± 8	2.6
2	2	2	2	111 ± 1	107 ± 6	59.2	40.8	91 ± 35	2.7
–	2	–	2	122 ± 3	82 ± 40	51.4	48.6	315 ± 67	2.1
–	2	–	–	119 ± 3	493 ± 85	54.9	45.1	190 ± 42	12.5
–	2	2	–	122 ± 3	488 ± 154	50.2	49.8	333 ± 94	12.4
–	–	2	2	127 ± 7	450 ± 138	56.4	43.6	211 ± 6	11.4
3	3	1	1	109 ± 1	45 ± 48	53.8	46.2	34 ± 8	1.1
–	3	1	–	127 ± 2	288 ± 194	60.0	40.0	131 ± 78	7.3
–	4	–	–	123 ± 2	595 ± 6	53.5	46.5	170 ± 27	15.1
–	–	4	4	125 ± 8	171 ± 157	56.4	43.6	170 ± 72	4.3
MH dosage (kg/ha)				**Plant height (cm)[a]**	**Grain yield (kg/ha)**	**Small sclerotia (%)**	**Primary sclerotia (%)**	**Total ergot yield (kg/ha)**	**Grain yield (%)**
Week of the year				**Hybrid rye L25P × L130N with imperfect male sterility**					
17th BBCH 45	**18th BBCH 47**	**19th BBCH 55**	**20th BBCH 59**						
–	–	–	–	115 ± 4	2822 ± 410	74.7	25.3	24 ± 13	100.0
2	–	2	–	95 ± 5	34 ± 27	54.4	45.6	14 ± 10	1.2
2	2	2	2	89 ± 4	51 ± 8	29.2	70.8	8 ± 2	1.8
2	2	2	–	90 ± 5	19 ± 14	36.0	64.0	5 ± 3	0.7
–	2	–	2	105 ± 2	160 ± 14	53.6	46.4	64 ± 38	5.7
–	2	–	–	107 ± 3	187 ± 6	57.1	42.9	83 ± 16	6.6
–	2	2	–	105 ± 2	202 ± 59	49.9	50.1	102 ± 5	7.2
–	–	2	2	112 ± 2	642 ± 152	61.2	38.8	53 ± 2	22.7

[a] Measured at phenological stage BBCH 69.

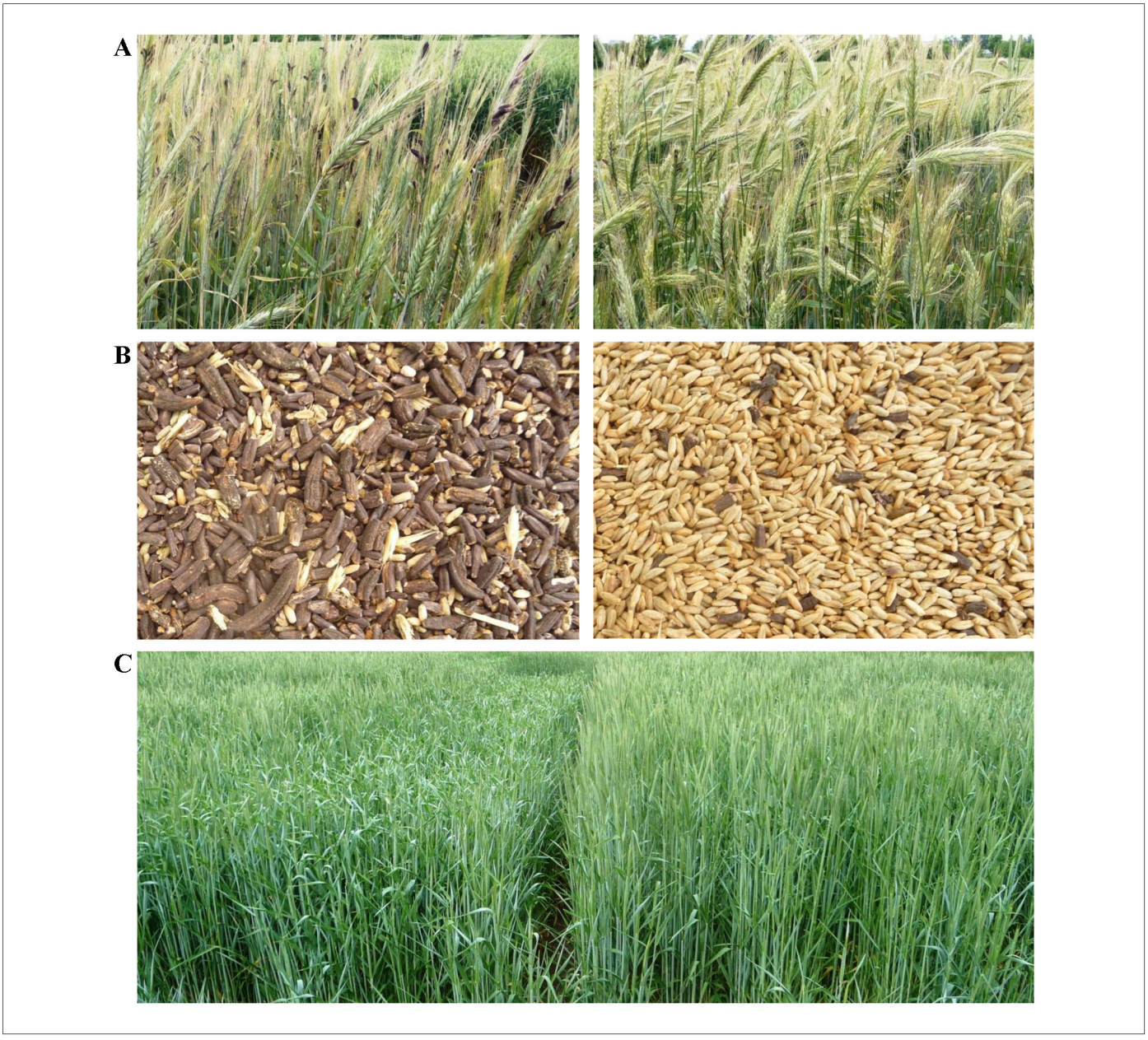

FIGURE 1

Effect of MH on the infection progress and ergot yield in the experiment from the 2013 season. **(a)** *C. purpurea* infection on the fertile rye variety Selgo treated with three dosages of MH (2 kg per ha, left) and the non-treated plot (right). **(b)** Ergot harvest from the plot with the fertile rye variety Selgo treated with MH (3 × 2 kg per ha, left) in contrast to the harvest from non-treated plants (right). **(c)** Effect of four subsequent applications of 1 kg of MH per ha on the rye hybrid L25P × L130N (left) in comparison with the non-treated plot (right).

application seemed to be crucial for obtaining a good ergot yield with minimal grain contamination. Therefore, the field experiment in the 2014 season was directed mainly to finding the proper application time.

Application time of maleic hydrazide to maintain high yield and low grain/ergot ratio

Trials with varied times of gametocide application demonstrated the great importance of proper timing. Even the lowest dosage of MH (1 kg per ha) caused a dramatic reduction in the ergot yield on both those varieties used when it was applied earlier than at the beginning of heading, the stage when awns became visible (Table 2). Earlier application caused more distinctive changes than did later application of even three times the lowest dosage. Strong reduction in plant height, darker leaf color, and a delay in inflorescence emerging from flag leaf sheath (Fig. 1c) were observed in plants treated at a phenological stage between BBCH 47 and 49, thus indicating that processes other than pollen germination (e.g. female gametophyte development) might also be affected. The negative effect of earlier application was observed in two consecutive seasons. The positive effect of more than one application is also obvious from both seasons' experiments. Generally, the

TABLE 4

***In vitro* testing of MH inhibitory effect on *C. purpurea* CCM 8405 conidia germination**

MH dosage (kg/ha)[a]	Germinated spores (%)	
	After 24 hours	After 48 hours
0	54	62
1	36	37
2	31	33
2.5	32	35
3	29	31
3.5	15	23
4	11	19

[a] At a concentration in the agar corresponding to an application of 400 L per ha.

application of 2 kg per ha in 3 subsequent weeks before the field is in full flowering, but after the ear emerges from the leaf sheath, appears to be the most promising. Lower ergot yield from the sterile variety in the 2014 season can be attributed to weather extremities. The spring of 2013 was extremely cold and wet with temperatures 0.8°C below and precipitation 116% above 30-year norms (1961–1990). On the other hand, the spring of 2014 had temperatures 2.4°C above and precipitation 86% below norms (Czech Hydrometeorological Institute; http://www.chmi.cz). In 2014, therefore, the development of rye plants was accelerated by approximately 2 weeks. As this estimation might not have been precise, the application of the first and second doses in 2014 was performed a couple of days earlier in relation to the plants' phenological stage. Moreover, fungal infections typically progress much better under cold and wet climate conditions [6], which occurred during the spring of 2013 (in which year higher yields were obtained).

Inhibitory effect of maleic hydrazide on the germination of C. purpurea *conidia*

The inhibitory effect of MH applied in the form of the commercial preparation Fazor® on *C. purpurea* spore germination was tested *in vitro* (Table 4). The results correspond well to the data obtained from the field experiments. Dosages of 1–3 kg of MH per ha showed only a weak negative effect on the ability of *C. purpurea* conidia to germinate. However, hyphae germinated from spores at MH concentrations above 3 kg per ha were significantly shortened. Nevertheless, if the time lag between infection and final gametocide application is not less than 5 days, the negative effect of the gametocide on spore germination can be ignored at a dosage around 2 kg per ha.

Ergot alkaloid content in sclerotia produced on rye treated with gametocides

Sclerotia grown on rye treated with MH and ETH during the 2014 experiment exhibited no significant alterations in EA content except for those that originated from plots with the earliest gametocide application (Table 5). Lower EA content in sclerotia obtained from plants where MH was applied at swollen flag leaf sheath stage was most probably due to strongly reduced growth of the plants and limited capacity of their sink. *C. purpurea* strain CCM 8405 is the main producer of the highly valued ergopeptine ergotamine, so it is noteworthy that the gametocides used in this work did not increase impurity content and ergotamine comprised 75–78% of total EA content. That content was similar to that obtained from sclerotia from non-treated plants. No significant

TABLE 5

Effect of selected gametocides on EA accumulation in sclerotia of *C. purpurea* CCM 8405. Ergotamine, ergotaminine, ergostine, ergostinine, 8-OH ergotamine, ergotoxine and ergotoxinine were quantified by ultra high performance liquid chromatography in the extract from a mixture of primary and small sclerotia. Presented are means ± SE of two biological replicates

Gametocide dosage (kg/ha)				Ergotamine[a] (% of dry mass)	% of control	Total EA (% of dry mass)	% of control
Week of the year				Sclerotia collected from the male-fertile rye Selgo			
17th	18th	19th	20th				
–	–	–	–	1.64 ± 0.28	100	2.09 ± 0.23	100
MH application							
–	2	–	2	1.71 ± 0.21	104	2.21 ± 0.27	106
3	3	1	1	1.11 ± 0.10	68	1.45 ± 0.10	69
–	3	1	–	1.52 ± 0.17	93	1.95 ± 0.21	93
–	–	4	4	1.44 ± 0.19	88	1.90 ± 0.15	91
ETH application							
3	–	3	–	1.69 ± 0.39	103	2.14 ± 0.40	102
1	–	1	–	1.50 ± 0.08	91	1.94 ± 0.11	93
Gametocide dosage (kg/ha)				Ergotamine[a] (% of dry mass)	% of control	Total EA (% of dry mass)	% of control
Week of the year				Sclerotia collected from the hybrid rye L25P × L130N			
17th	18th	19th	20th				
–	–	–	–	1.42 ± 0.22	100	1.82 ± 0.23	100
MH application							
2	2	2	2	0.90 ± 0.21	63	1.16 ± 0.30	64
–	2	–	2	1.33 ± 0.22	94	1.76 ± 0.26	97

[a] Sum of ergotamine and its oxidative product ergotaminine.

differences in EA composition and quantity were observed between primary and secondary sclerotia (data not shown).

Conclusion

Streamlining the ergot field production process can lead to a significant reduction in costs associated with manufacturing EA-based drugs. Male sterility that is chemically induced via an appropriate gametocide might minimize the impact of shortcomings in agro-technical practice. The procedure proposed in this work decreases the contents of organic contaminants coming from rye grain in the harvested sclerotia mass, but most importantly it permits the producer to use common fertile rye cultivars. The method of maintaining a population of fully sterile rye and preparing hybrid seed stock is challenging, and every failure by the human operator is followed by a large increase in undesirable grains contaminating the ergot. That means the costs of high-quality male-sterile hybrid seeds are as much as 20 times greater than are those of standard fertile rye cultivars. Plant protection products containing MH, an auxin antagonist with gametocide effect that was found to be the most promising, are readily available and relatively inexpensive. Thus, their application needs not significantly increase the costs of EA field production. Because *C. purpurea* is a living organism, sclerotia yield always varies from season to season, depending mainly on climatic conditions, as is also evident from the differing results obtained in the three subsequent seasons presented herein. Nevertheless, employing the gametocide can significantly increase the purity of the ergot while having little or no impact on its total yield if the application is accurately timed. Teva Czech Industries is presently one of the world's largest producers of EA-based drugs. The company's production is centered mainly on parasitic field cultivation and has generated undesirable losses over the past decade due to the heterogeneity of hybrid rye host plants. The first attempt at large-scale ergot cultivation on a fertile rye variety treated with gametocide brought a promising improvement in the cost-effectiveness of EA production for the 2014 season. Preliminary estimates of ergot yield range between 0.5 and 1 ton per hectare with organic impurities up to 30% for production at two independent locations (2 and 3 ha areas) using a fertile rye variety treated with two dosages of MH.

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PALACKY UNIVERSITY OLOMOUC

Faculty of Natural Sciences

Department of Biochemistry

Ph.D. Thesis summary

GENETIC MODIFICATION OF *CLAVICEPS PURPUREA* AND ERGOT ALKALOIDS PRODUCTION

P1406 Biochemistry

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Olomouc

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This Ph.D. thesis has been completed at the Division of Molecular Biology in the Department of Biochemistry, Faculty of Science, Palacký University Olomouc under the supervision of Doc. Mgr. Petr Galuszka, Ph.D.

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The oral defence will take place at the Faculty of Science, Šlechtitelů 11, Olomouc on……

The Ph.D. thesis is available at the Library of the Biological Centre, Šlechtitelů 11, Olomouc.

Prof. RNDr. Ivo Frébort, Ph.D.
Chairman of Doctoral Study Board in Biochemistry

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3. Constitutive, strong expression of selected genes of the pathway of biosyntheses of ergot alkaloids and testing of its impact on quality and quantity of produced alkaloids

4. Optimalization of method of field production of ergot alkaloids

Abstract

Presented thesis is focused on study of ergot alkaloids and on affecting of their production in *Claviceps purpurea* and is divided into three parts.

The first part of the thesis is devoted to the literature review focused on biology of the *Claviceps purpurea*, genetics of ergot alkaloids production, their chemical structure and resulting biological activity. Methods of industrial production of ergot alkaloids, methods of genetic transformation of the *Claviceps purpurea* and possibilities for affecting of the ergot alkaloids production by means of molecular biology techniques are described.

The second part of the thesis is focused on optimalization of the field production of ergot alkaloids by the gametocide application. Yield of sclerotia can be significantly reduced by presence of pollen contamination originating from imperfect male-sterile rye, as only unfertilized ovaria can be infected by spores of *Claviceps purpurea*.

Application of suitable gametocide leads to significant improvement of rye male-sterility of both, a fertile rye variety and a variety with imperfectly induced cytoplasmic male sterility.

Maleic hydrazide was proven to be a highly effective gametocide for both of the above-mentioned variety of rye and showed negligible effect on germination of *C. purpurea* spores. Both accurate dosaging of the active gametocidal compound and timing of the application just 2–3 weeks before onset of anthesis proved crucial to achieving high ergot yield with minimum grain impurities.

The third part of the thesis is focused on genetic background of the production of ergopeptides in industrial strains of *C. purpurea*. Optimized method for transformation of *C. purpurea* was introduced at the department of Molecular Biology, Centre of region Haná with kind help of Prof. Tudzynski´s group and provides large number of transformants per transformation. The method was used for generation of two independent *LpsA2* knock-out mutants, selected from total of 230 transformants. Chemical analysis of the knock-out mutants showed degeneration of the strain, resulting in loss of the ability to produce ergot alkaloids in the knockout mutants, as well as in the transformants with an ectopically integrated knockout cassette.

Three genes of cluster for biosynthesis of ergot alkaloids (ie. *dmaW, easC* and

easG) were selected as potential limiting steps of the pathway, determining the rate of the production of ergot alkaloids. Expression of selected genes under the control of strong, constitutive promoter gpdA didn´t lead to any biotechnologically significant, sustainable changes in EA production of tested strains.

The *LpsA1* gene, responsible for the production of ergotamine in the 20.1 strain, was constitutively expressed in the Gal 130 strain, the ergocristine producer. Two independent Gal 130 OE *LpsA1*, 20.1 transformants were analyzed for qualitative changes in their ergot alkaloid production. A significant improvement in ergotamine representation, which is originally the minor alkaloid of the strain, representing in WT only around 1% of the EA pool, was observed in both of the mutants.

Abstrakt

Předkládaná disertační práce je zaměřená na studium a ovlivňování produkce námelových alkaloidů organismem *Claviceps purpurea* a sestává ze tří částí.

V první části disertační práce je vypracována odborná rešerše, zabývající se biologií organismu *Claviceps purpurea*, genetickým podkladem produkce námelových alkaloidů, jejich chemickou strukturou a z ní vyplývající biologickou aktivitou. Jsou popsány metody průmyslové produkce námelových alkaloidů, metody genové transformace organismu *C. purpurea* a možnosti ovlivnění produkce metodami molekulární biologie.

Druhá část disertační práce je zaměřená na optimalizaci polní produkce námelových alkaloidů aplikací vhodného gametocidu. Výnos sklerocií může být významně redukován pylem pocházejícím z nedokonale pylově sterilního žita, jelikož pouze neoplodněná ovaria mohou být sporami *C. purpurea* infikována. Aplikací vhodného gametocidu dojde k významnému zlepšení sterility žita s nedokonale navozenou cytoplasmatickou sterilitou či indukci sterility u fertilního žita. Maleinhydrazid prokázal vysokou míru gametocidního účinku u obou variant žita, přičemž měl zanedbatelný efekt na klíčení spor *C. purpurea*. Optimální čas aplikace (2-3 týdny před antézou) i optimální dávkování byly klíčové pro zisk vysokého množství sklerocií při minimálním znečištění zrnem.

Třetí část disertační práce byla zaměřena na genetický podklad produkce ergopeptidů v průmyslových kmenech *C. purpurea*. Na Oddělení Molekulární biologie, Centra regionu Haná, Palackého university byla za laskavé pomoci skupiny Prof. Tudzynského zavedena optimalisovaná metoda transformace *C. purpurea*, zajišťující zisk velkého množství transformantů za transformaci. Metoda byla použita k přípravě dvou nezávislých *LpsA2* knock-out mutantů vyselektovaných z množství 230 transformovaných kolonií. Chemická analýza knock-out mutantů stejně jako vzorků s náhodně integrovanou deleční kazetou prokázala degeneraci kmene a z ní vyplývající neschopnost produkce námelových alkaloidů.

Tři geny klastru pro biosyntézu námelových alkaloidů (tj. *dmaW, easC* a *easG*) byly vybrány jako potenciální limitující kroky dráhy, určující míru produkce námelových alkaloidů. Jejich exprese pod silným konstitutivním promotorem gpdA nezapříčinila významné a udržitelné zvýšení produkce námelových alkaloidů v testovaných kmenech.

Gen *LpsA1,* odpovědný za produkci ergotaminu v kmeni 20.1 byl konstitutivně exprimován v kmeni Gal 130, produkujícím ergokristin jako hlavní alkaloid. Dva nezávislí Gal 130 OE *LpsA1*, 20.1 transformanti byli testováni pro kvalitativní změny v produkci námelových alkaloidů. Bylo u nich zjištěno významné zvýšení relativního zastoupení ergotaminu, jež je ve WT kmeni Gal 130 produkován v míře přibližně 1% z celkového obsahu námelových alkaloidů.

Part I – General Introduction - Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids

1.1 Abstract

Ergot alkaloids produced by the fungus *Claviceps* parasitizing on cereals, include three major groups: clavine alkaloids, D-lysergic acid and its derivatives and ergopeptines. These alkaloids are important substances for the pharmatech industry, where they are used for production of anti-migraine drugs, uterotonics, prolactin inhibitors, anti-Parkinson agents, etc. Production of ergot alkaloids is based either on traditional field cultivation of ergot-infected rye or on submerged cultures of the fungus in industrial fermentation plants. In 2010, the total production of these alkaloids in the world was about 20,000 kg, of which field cultivation contributed about 50%. This review covers the recent advances in understanding of the genetics and regulation of biosynthesis of ergot alkaloids, focusing on possible applications of the new knowledge to improve the production yield.

1.2 Introduction

The *Clavicipitaceae* family includes, among others, a fungal species called ergot that is important for humankind, in terms of both contributions and losses. In medieval history, these fungi parasitizing in cereals and producing a range of ergot alkaloids caused many mass poisonings that resulted in painful deaths of tens of thousands of people (Schiff, 2006). Nowadays, mass poisonings with ergot alkaloids are sporadic and occur only in developing countries. For example, an epidemic of ergotism with a high mortality rate occurred in 1977 in Ethiopia (Demeke et al., 1979). Contrary to mass poisonings, the recent cases of individual poisonings are usually connected with overdoses of medical drugs based on these alkaloids.

For thousands of years, people tried to take advantage of substances produced by ergot without knowing their chemical structure, proper dosage or side effects, but scientific research on ergot alkaloids started only with the beginning of modern pharmacy in the early 20th century. Nowadays, specific types of ergot alkaloids are widely used as a basic drug-stock for the production of various therapeutic substances, e.g. for the treatment of migraine, in gynecology for its uterotonic effects, as prolactin inhibitors, antiparkinsonian drugs, etc. (de Groot et al., 1998).

The *Claviceps* genus is not only interesting for its ability to produce secondary metabolites usable in pharmaceutical industry, but also in its life strategy, mainly the specificity of invaded host organs, which is in the center of interest of many scientific groups. Last, but

not least, its negative influence in agriculture should be mentioned. Some of the *Claviceps* species cause significant losses in cereal production. Recently, there was an infection of the fifth most important cereal crop in the world, *Sorghum bicolor* (Haarmann et al., 2009), by *Claviceps africana*.

1.3 Ergot Alkaloids

1.3.1. Chemistry and occurrence of ergot alkaloids

Ergot alkaloids belong to the class of indole derivatives. They can be divided into three major groups: clavine alkaloids, D-lysergic acid and its simple derivatives and ergopeptines. Structures of typical ergot alkaloids are shown in Fig. 1. Species within the genus *Claviceps* differ in their capability to produce diverse types of alkaloids. Only a few species (e.g. *Claviceps purpurea* and *C. africana*) can produce ergopeptines as final products of their ergot alkaloid biosynthetic pathway. For example, the biosynthetic pathway of *Claviceps fusiformis* ends with elymoclavine production that has been explained as a loss of the late pathway genes in the ergot alkaloid gene cluster (Lorenz et al., 2007).

Most of ergot alkaloids contain a tetracyclic ergoline structure, although some of the naturally occurring clavine alkaloids are tricyclic, e.g. chanoclavine-I, chanoclavine-II and isochanoclavine-I (for a review see Buchta and Cvak, 1999). Of these, only chanoclavine-I can serve as a precursor for biosynthesis of other ergot alkaloids. Tetracyclic clavine alkaloids can be found in various species of fungi. Agroclavine and elymoclavine are intermediates of the pathway leading to D-lysergic acid production, but in most ergot alkaloid producing fungi, this pathway ends with clavine alkaloids as final products.

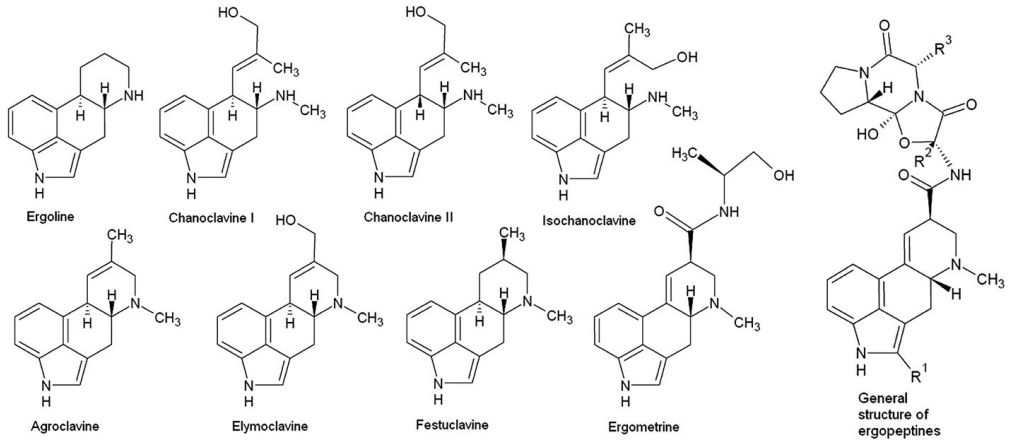

Fig. 1. Structures of ergot alkaloids.

1.3.2. Biosynthesis of ergot alkaloids: enzymology and genetics

By chromosome walking, a whole 68.5 kb EAS cluster, consisting of 14 genes (Fig. 2C), was described by Paul Tudzynski's group (Haarmann et al., 2005).

The general biosynthetic pathway of ergot alkaloids is shown in Fig. 2A,B. As precursors of the ergoline system, three primary metabolites were found: tryptophan, a product of the mevalonate pathway dimethylallylpyrophosphate (DMAPP) as a donor of the isoprene unit, and methionine as a methyl group donor.

The first specific step of the biosynthetic pathway is the isoprenylation of tryptophan resulting in 4-(γ,γ-dimethylallyl)tryptophan (DMAT). This step, catalyzed by 105 kDa homodimer enzyme DMAT synthase (Gebler and Poulter, 1992), is positively regulated by tryptophan and negatively feedback-regulated by agroclavine and elymoclavine, further products of the biosynthetic pathway (Cheng et al., 1980). The second step of the EAS pathway is the methylation of DMAT to *N*-methyl-dimethylallyltryptophan (MeDMAT). The biosynthetic pathway continues with several oxidation and reduction steps resulting in the formation of chanoclavine I, chanoclavine I aldehyde and then agroclavine. In *C. purpurea*, chanoclavine synthase is encoded by *ccsA* gene of 1503 bp, originally named *easE* (Lorenz et al., 2010). Chanoclavine-I is then further oxidized to chanoclavine-I aldehyde by an alcohol dehydrogenase encoded by *easD* (Wallwey and Li, 2011).

Chanoclavine-I aldehyde first undergoes double bond isomerization to isochanoclavine-I aldehyde, originally thought to be catalyzed by the product of *easA* gene. It was shown recently that the reaction also proceeds non-enzymatically by reduction with glutathione or 2-mercaptoethanol (Matuschek et al., 2011). Isochanoclavine-I aldehyde then forms an iminium ion compound, which is subsequently reduced to agroclavine by the enzyme easG (agroclavine synthase) in the presence of NADPH. The monomeric easG is encoded by *easG* gene of 1079 bp containing two introns (Matuschek et al., 2011). The biosynthetic pathway then continues with a cloA catalyzed oxidation of agroclavine on C17 atom, leading to formation of elymoclavine. Elymoclavine is oxidized to paspalic acid again by clavine oxidase cloA (Haarmann et al., 2006; Robinson and Panaccione, 2014). CloA protein is encoded by *cloA* gene of 2120 bp interrupted with eight introns (Haarmann et al., 2006). Paspalic acid is converted to D-lysergic acid spontaneously, as described by Gröger and Floss (1998).

D-lysergic acid is captured and activated by monomodular non-ribosomal peptide synthetase (NRPS) LPS2, one of the two subunits of D-lysergyl peptide synthetase with a molecular mass of 141 kDa. D-lysergic acid is, after binding to LPS2 as a thioester, transferred to trimodular LPS1 NRPS, the second subunit of D-lysergyl peptide synthetase with a size of 370 kDa. Here it is condensed with three bound amino acids, resulting in formation of a D-lysergyl tripeptide lactam (Walzel et al., 1997). Finally, D-lysergyl peptide lactam is oxidized by easH (Havemann et al., 2014) and the product is spontaneously converted to ergopeptines (Haarmann et al.,2006).

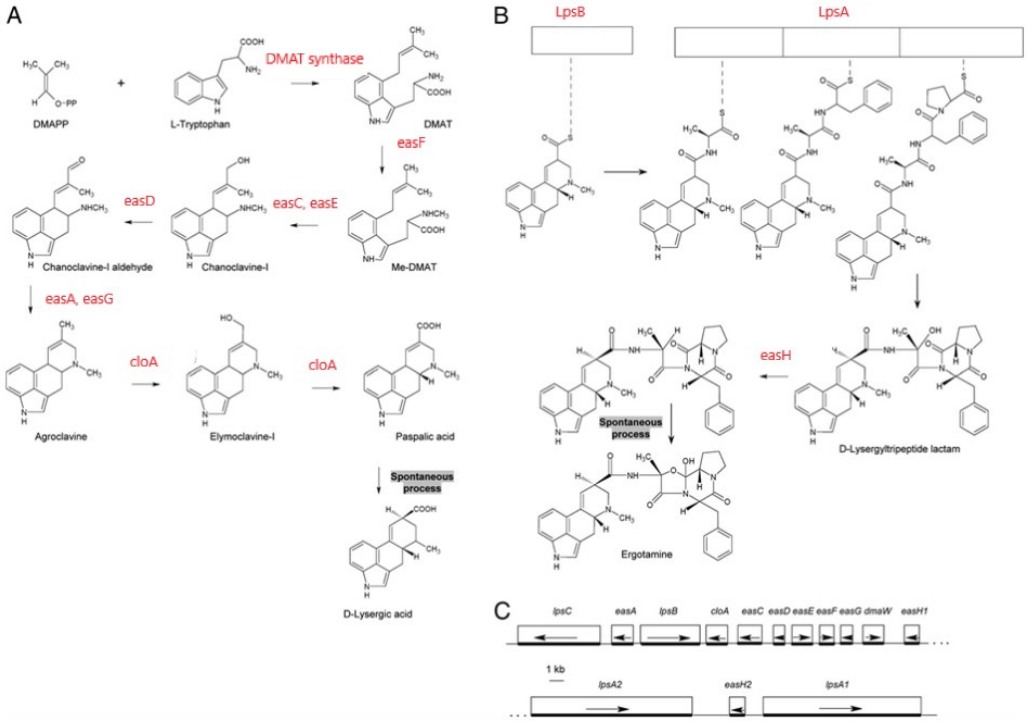

Fig. 2. Biosynthesis of ergot alkaloids in *Claviceps purpurea*: A) scheme of the biosynthetic pathway leading to D-lysergic acid, B) biosynthesis of ergopeptines on two-component non-ribosomal peptide synthetase (NRPS), and C) scheme of the ergot alkaloid synthesis (EAS) gene cluster.

1.3.3. Biological activity of ergot alkaloids

Specific types of ergot alkaloids serve as a basic drug-stock for production of various therapeutic substances to treat migraine headaches, Parkinson's disease, hypertension and diverse sexual disorders (de Groot et al., 1998). Biological activity of ergot alkaloids relates to their structural similarities with neurotransmitters noradrenalin, dopamine and serotonin. The major pharmaceutical effects of ergot alkaloids are smooth muscle stimulation, central sympatholytic activity and peripheral α1-adrenergic blockade. Smooth muscle stimulation is the most evident as a vasoconstriction and uterine contraction (Innes, 1962). Anti-migraine effects of many ergot alkaloids are facilitated by interactions with serotonin receptors, 5-HTs. First compound used for the treatment of this disorder was ergotamine in 1926 (Maier, 1926). The main disadvantage of natural ergot alkaloids is a lack of selectivity for each individual 5-HT receptor, which stimulated the development of semisynthetic serotonergic ligands that are more selective (Pertz and Eich, 1999).

1.4 Production of ergot alkaloids for pharmaceutical industry

1.4.1. The fungus *Claviceps*

C. purpurea, is spread worldwide and has the widest host range of any *Claviceps* species. It can infect about 400 species of grasses (Taber, 1985), but its most typical hosts are rye, wheat and barley (Loveless, 1971).

The sexual life cycle starts with an infection of unfertilized rye ovaries. Specialized penetration structures are not known in *C. purpurea*, indicating that the cell wall is passed due to enzyme secretion (Haarmann et al., 2009). Conidia are transported to the plant by wind or insects, the plant cuticle is penetrated and fungal hyphae colonize ovarian tissues. After 5 to 7 days post infection, the first macroscopically visible sign of infection, production of honeydew, can be detected. The honeydew production stops after about 2 weeks being followed by the development of a rigid stage sclerotium, composed of a compact mass of hardened fungal mycelium (Tenberge, 1999; Tudzynski and Scheffer, 2004) as shown in 3C. After 5 weeks post infection, sclerotia mature (Kirchhoff, 1929). Sclerotia can survive unfavorable conditions in winter and germinate after a temperature increase in the spring (optimally at about 20 °C) on or beneath the soil surface (Kirchhoff, 1929), forming stromata composed of stalks with spherical capitula that grow in a phototropic manner to reach the air (Hadley, 1968). In nature, sclerotia are the unique structures, which produce all types of ergot alkaloids (Ramstad and Gjerstad, 1955).

1.4.2. Field production on rye

The world production of ergot alkaloids reaches thousands of kilograms annually that include both ergopeptines and semisynthetic ergot alkaloid derivatives, e.g. cabergoline and pergolide (Cvak, 1999). In 2010, the total world production was about 20,000 kg, into which the field cultivation contributed about 50% (Vít Kubesa, Teva Czech Industries, Opava, Czech Republic; personal communication).

The traditional method of ergot production is based on spraying a conidial suspension onto a field-cultivated rye (*Secale cereale*). From the 1990s, hybrid rye lines with induced male-sterility, such as *Hyclaro* (Rentschler Biotechnologie, Laupheim, Germany), were introduced as the host because their unpollinated florets stay open longer and thus extend the period of susceptibility to ergot infection, which leads to a significantly better yield of generated sclerotia (Németh, 1999).

An important benefit of the field ergot production is the wide variability of strains available for the production of specific types of alkaloids and their better genetic stability compared to the mutant strains used for submerged cultures. The yield of field production can reach 1–2 tons of sclerotia and from 10 kg (Tudzynski et al., 2001) to 20 kg (TEVA Czech Industries, in present) of ergot alkaloids per hectare. However, the field production can be

dramatically influenced by climatic conditions of the particular year as well as by the quality and uniformity of used hybrid rye.

1.4.3. Production in fermentation plants

Only some selected mutant strains have the ability to produce alkaloids in submerged cultures; the production strategies were extensively reviewed by Malinka (1999). Cells with this ability, which are called sclerotia-like cells, are shorter and thicker than the normal ones with a robust cell wall and large vacuoles (Spalla, 1973). However, a degeneration process that leads to a loss of the sclerotia-like cell morphology and lowered alkaloid production is relatively frequent; therefore a continuous selection is needed to maintain a good production strain (Malinka, 1999).

Fermentation is nowadays used mostly to produce paspalic acid, which isomerizes to D-lysergic acid, and some derivatives of paspalic acid that serve as a starting material for preparation of semisynthetic alkaloid derivatives. D-lysergic acid can be also obtained by alkaline hydrolysis of simple amides, ergometrine and even ergopeptines (Rucman, 1976).

Production of ergopeptines by fermentation is much more complicated and requires specific conditions.

Biosynthesis of ergot alkaloids in axenic cultures is positively regulated by tryptophan, which acts as a precursor and inducer, and negatively by phosphate and ammonium, which repress biosynthesis (Sočič and Gaberc-Porekar, 1992).

1.4.4. Genetic transformation of *Claviceps*

Undoubtedly, the most popular method for the genetic transformation of *Claviceps* is protoplast transformation in a medium of high osmotic pressure. A successful method based on protoplast preparation using lytic enzymes was first described in 1989 (Engelenburg et al., 1989) and was later improved (Mey et al., 2002). There are many commercially available enzymes or their mixtures suitable for *C. purpurea* protoplast preparation, such as Lysing enzyme and Driselase (Mey et al., 2002) or β-glucuronidase (Keller et al., 1980). Ca^{2+} ions are universal component of the transformation mixture, while the high osmotic pressure can be adjusted by adding PEG (Engelenburg et al., 1989; Mey et al., 2002). Transformants can then be selected based on acquired antibiotic resistance (Engelenburg et al., 1989; Mey et al., 2002) or by using pyrimidine auxotrophy (Smit and Tudzynski, 1992). Hygromycin (1.0 mg/ml; Comino et al., 1989) and phleomycin or bleomycin (0.1 mg/ml; Mey et al., 2002) are the most often used selection antibiotics. Selective growth of transformed vs. untransformed mycelia on a medium containing phleomycin is shown in 3D (Hulvová et al., 2009).

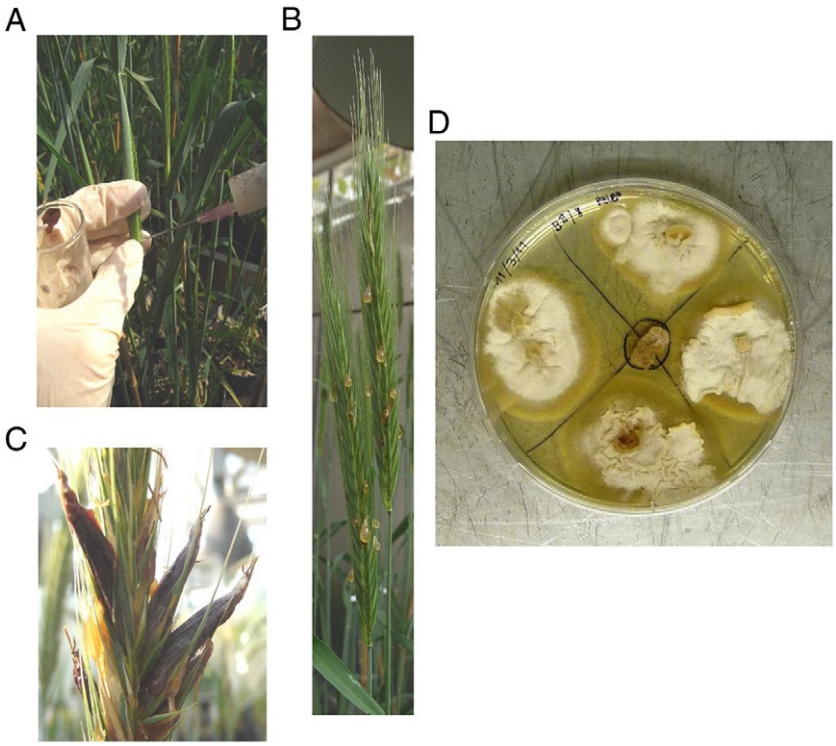

Fig. 3. Various stages of the growth of *Claviceps purpurea*: A) inoculation of rye with conidia suspension, B) honeydew production on rye, C) matured sclerotia growing on rye, D) selection of *Claviceps purpurea* transformants on a Petri dish: protoplasts of the industrial strain GAL404 were transformed with a linearized plasmid bearing *ble* gene conferring phleomycin resistance; a selective growth of transformed mycelia (on the plate periphery) contrary to untransformed mycelia (in the center) on BII agar plate with 100 µg/ml phleomycin.

Part II - Improving field production of ergot alkaloids by application of gametocide on rye host plants

2.1 Abstract

Ergot alkaloids are widely used in the pharmaceutical industry in drug preparations for treating migraines and Parkinson's disease, inducing uterine contraction, and other purposes. Phytopathogenic fungi of the genus *Claviceps* (e.g. *C. purpurea*) comprise a major biological source of ergot alkaloids. Worldwide industrial production of these alkaloids derives almost equally from two biotechnological procedures: submerged culture of the fungus in fermenters and field parasitic production in dormant fungal organs known as sclerotia (also termed ergot).

Ergot yields from field cultivation are greatly affected by weather and also can be much reduced by pollen contamination from imperfectly male sterile rye, as only unfertilized ovaries can be infected by *C. purpurea* spores.

Two substances with gametocidal effect – maleic hydrazide and

2-chloroethylphosphonic acid – were tested during three consecutive seasons in small field experiments for the ability to induce or amplify the male sterility of rye as well as the impacts on germination of *C. purpurea* spores and general vitality of rye host plants. Maleic hydrazide was proven to be a highly effective gametocide on both a fertile rye variety and a variety with imperfectly induced cytoplasmic male sterility. It showed negligible effect on germination of *C. purpurea* spores. Both accurate dosaging of the active gametocidal compound and timing of the application just 2–3 weeks before onset of anthesis proved crucial to achieving high ergot yield with minimum grain impurities.

2.2 Introduction

Most *Claviceps* species and strains are able to produce EAs only during their parasitic lives, and that is especially true of those producing ergopeptines as a final product. Industrial strains of *C. purpurea* producing sclerotia containing large amounts of ergopeptines have been obtained through long-term breeding and random mutagenesis. These strains can accumulate as much as 2% of EA in the dry weight of sclerotia. Ergot yields become economically profitable at around one-half ton per hectare of infected rye field (Tudzynski et al., 2001; Ne´meth, 1999) (Teva Czech Industries, recent years; unpublished results).

Parasitic production in the field is based on spraying or injection of non-pollinated rye plants in the earing phase with a conidial suspension of *C. purpurea*. During the 1980s, introduction of hybrid rye lines with cytoplasmic male sterility, such as the cultivar Hyclaro (Rentschler Biotechnologie, Laupheim, Germany) contributed to significantly increased ergot yield (Ne´meth, 1999). These hybrid lines produce no pollen; their florets stay open for a longer period and are thus more susceptible to the infection. Nevertheless, there exist many additional factors which can negatively affect field production. In addition to climatic conditions, the quality and homogeneity of hybrid seed stock are crucial. Even a tiny amount of contamination with the parental component during hybrid seed stock preparation can produce a sufficient amount of pollen on the production field to cause undesirable formation of grains. Sclerotia formation is then reduced, and that in turn significantly increases the cost of drug preparation.

For cereals and other crops, male sterility can be also induced chemically. A gametocide is a morphogenetic poison that is typically used to treat the plants in the stage of generative organ initiation (Vorob'ev et al., 2005).

Depending on dosage, gametocides can cause partial or full male sterility. Determining the appropriate dose is very important, as higher doses can also negatively affect plant growth and the development of female reproductive organs.

Schoene and Hoffman (Schoene and Hoffman, 1949) first described the inhibitory effect of maleic hydrazide (MH; 1,2-dihydro-3,6-pyridazinedione) on plant growth. Later, the compound was successfully used as a male gametocide for many plant species, including coriander (Kalidasu et a., 2009), rice (Lakshmi Praba and Thangaraj, 2005), and wheat (Chopra et al., 1960). MH is an auxin and gibberellic acid agonist (Brian and Hemming, 1957) that is commonly used as a diethanolamine salt or sodium salt soluble in water.

Ethrel has been studied for gametocidal effect in many plant species. It is used as a convenient gametocide for wheat, wherein it adequately induces male sterility while maintaining female fertility when applied at an appropriate pre-meiosis growth stage (Sharma and Sharma, 2005). The aim of the present work was to study the effectiveness of gametocides on the parasitic production of ergot in a series of field experiments.

2.3 Materials and Methods

Biological material, rye field preparation, conidiospor preparation and field infection, harvest and yield evaluation, spore inhibition assay and ergot alkaloids quantification are subscribed in more detail in Hanosová et al., 2015.

2.4 Results and discussion

2.4.1. Selection of gametocide with positive effect on ergot yield

Plant protection products commercially available and certified for use in the European Union were surveyed for the presence of active components having gametocidal effect. Finally, two products containing ETH and MH as active compounds, respectively, were selected. These were Cerone®, which is used to increase cereal resistance to lodging, and Fazor®, which is used as a sprouting suppressor for potato tubers or onion bulbs. In the 2012 season, small experimental plots with the hybrid male-sterile rye L25P x L130N were treated with doses ranging from 2 to 15 kg and 2.5 to 160 kg per ha of MH and ETH, respectively, 3 weeks before onset of flowering (BBCH 55, Lancashire et al., 1991). Higher dosages of ETH (above 20 kg per ha) showed such immediate negative effects as formation of leaf lesions, leaf rolling, and cessation of growth and ear development that were visible already a few days after the application. None of these effects were observed on plants treated with MH. Both gametocides caused reductions in plant height across the whole range of dosages used (Table 1).

Dosage (kg/ha) applied at BBCH 51	Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
None	82 ±2	2750 ±308	42.9	57.1	1395 ±106	100.0
Maleic hydrazide						
2	69 ±1	26 ±6	22.9	77.1	484±32	0.9
4	69 ±2	6 ±2	42.6	57.4	193±21	0.2
8	69 ±1	3 ±0	44.4	55.6	127±100	0.1
15	59 ±1	None	None	None	None	0.0
Ethrel						
2.5	64 ±2	1730 ±252	80.7	19.3	674±48	62.9
5	60 ±1	1994 ±704	75.5	24.5	824±93	72.5
10	61 ±1	102±16	66.2	33.8	302±12	3.7
20	55 ±1	86±22	38.8	61.2	294±32	3.1
40	57 ±1	202±41	54.5	45.5	132±29	7.3
80	52 ±1	130±40	50.9	49.1	110±7	4.7
160	51 ±1	74±30	100.0	None	16 ±4	2.7

Table 1. Effects of different dosages of MH and ETH on grain production and ergot yield on hybrid rye L25P x L130N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2012 season).

After treated plants as well as non-treated controls were infected by *C. purpurea* conidia, the progress of infection was followed and ergot yield estimated. The first symptoms of infection – formation of honeydew droplets and polyamine odor – were obvious 12 days after infection with the same intensity on control plots as on plots with gametocide dosages up to 4 kg and 20 kg per ha for MH and ETH, respectively. Plots with higher dosages showed significantly lower infection symptoms. The seed stock used (hybrid rye L25P x L130N) showed a high penetration of fertile individuals ranging from 2% to 20%. MH significantly decreased grain yield for treated plants already at a dosage of 2 kg per ha in contrast to non-treated plants (Table 1). ETH treatment caused a similar reduction, but only at much higher doses and while also negatively affecting plant height and vitality already at a dosage of 2.5 kg per ha. Although both gametocides also slightly reduced ergot yield even at the lowest application dosages, the decrease in contaminating rye grains in the total harvest was far more prominent, and especially so in the case of MH. In contrast to ETH, MH did not substantially affect plant vitality or growth. MH was therefore selected for further testing in subsequent seasons. ETH treatment was repeated in the 2014 season with similar results. There was a proportional reduction in plant height and grain and ergot yield with increasing ETH dosage from 1 to 5 kg per ha (data not shown), and the total ergot yield and grain/ergot ratio were much better when MH was used.

2.4.2. Ergot yield from fertile rye variety treated with maleic hydrazide

Although male-fertile rye varieties have not been used for industrial production in recent decades, some of them have high susceptibility to *C. purpurea* infection, and especially those with a broader range of flowering time and longer ears. It is crucial to infect fertile varieties before pollination onset as the pollen competes strongly with ergot conidia (Ne´meth, 1999). Since rye is cross-pollinating and the onset of flowering in the field is not synchronized, a huge amount of pollen transmitted by wind from early maturing individuals can block *C. purpurea* infection of those ovaries that were not infected early enough.

The fertile rye variety Selgo was treated with MH at different time points, beginning 3 weeks before the onset of flowering. Similarly to the case of the male-sterile variety, MH application visibly affected only plant height while not altering flower development and pollen shedding from anthers. The fertile rye was not inoculated until phenological stage BBCH 62, at which time approximately 30% of the anthers in the field were mature and produced pollen. Despite pollen production, decrease in grain formation by more than 10-fold was observed on all plots where the gametocide was applied (Tables 2 and 3). The MH concentrations used and the application during the period when male gametophytes are already in development most probably did not affect microspore formation. Rather, these led to the formation of pollen grains that were unable to germinate.

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Hybrid rye L25P x L130N with imperfect male sterility					
19th BBCH 47	20th BBCH 49	21th BBCH 55	22th BBCH 59						
–	–	–	–	119 ± 6	1938 ± 370	63.7	36.3	178 ± 22	100.0
–	–	0.5	0.5	112 ± 2	844 ± 71	39.3	60.7	164 ± 114	43.6
–	0.5	–	–	113 ± 2	2245 ± 254	67.0	33.0	197 ± 18	115.8
1	1	1	1	77 ± 1	17 ± 12	66.3	33.7	74 ± 7	0.9
–	1	1	1	116 ± 1	487 ± 354	60.4	39.6	624 ± 72	25.1
–	1	1	–	113 ± 2	1317 ± 175	89.5	10.5	295 ± 19	68.0
–	1	–	–	105 ± 2	1168 ± 369	85.7	14.3	91 ± 9	60.3
–	–	1.5	1.5	108 ± 6	137 ± 48	61.5	38.5	670 ± 125	7.1
–	1.5	–	–	110 ± 2	1078 ± 87	93.5	6.5	217 ± 14	55.6
–	2	2	2	106 ± 1	90 ± 57	78.9	21.1	462 ± 49	4.6
–	2	–	–	108 ± 3	371 ± 154	83.9	16.1	193 ± 15	19.1
–	3	–	–	103 ± 3	197 ± 35	59.4	40.6	271 ± 32	10.2
MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Fertile rye Selgo					
19th BBCH 49	20th BBCH 51	21th BBCH 59	22th BBCH 61						
–	–	–	–	124 ± 3	4301 ± 136	61.1	38.9	190 ± 11	100.0
1	–	1	1	97 ± 3	7 ± 2	35.7	64.3	14 ± 2	0.2
–	1	1	1	118 ± 3	416 ± 113	24.7	75.3	308 ± 38	9.7
–	2	2	2	105 ± 4	114 ± 53	42.1	57.9	428 ± 58	2.7
–	3	3	3	106 ± 1	209 ± 112	27.6	72.4	485 ± 392	4.9

[a] Measured at phenological stage BBCH 69.

Table 2. Effects of different MH dosages and application times on grain production and ergot yield for hybrid rye L25P x L130N with imperfect male sterility and fertile rye variety Selgo infected by *C. purpurea* CCM 8405 (experiment run during the 2013 season).

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield
Week of the year				Fertile rye Selgo					
17th BBCH 49	18th BBCH 51	19th BBCH 59	20th BBCH 61						
–	–	–	–	127 ± 6	3946 ± 957	58.6	41.4	162 ± 109	100.0
2	–	2	–	110 ± 1	38 ± 29	38.5	61.5	85 ± 61	1.0
2	2	2	–	106 ± 8	101 ± 22	54.2	45.8	69 ± 8	2.6
2	2	2	2	111 ± 1	107 ± 6	59.2	40.8	91 ± 35	2.7
–	2	–	2	122 ± 3	82 ± 40	51.4	48.6	315 ± 67	2.1
–	2	–	–	119 ± 3	493 ± 85	54.9	45.1	190 ± 42	12.5
–	2	2	–	122 ± 3	488 ± 154	50.2	49.8	333 ± 94	12.4
–	–	2	2	127 ± 7	450 ± 138	56.4	43.6	211 ± 6	11.4
3	3	1	1	109 ± 1	45 ± 48	53.8	46.2	34 ± 8	1.1
–	3	1	–	127 ± 2	288 ± 194	60.0	40.0	131 ± 78	7.3
–	4	–	–	123 ± 2	595 ± 6	53.5	46.5	170 ± 27	15.1
–	–	4	4	125 ± 8	171 ± 157	56.4	43.6	170 ± 72	4.3

MH dosage (kg/ha)				Plant height (cm)[a]	Grain yield (kg/ha)	Small sclerotia (%)	Primary sclerotia (%)	Total ergot yield (kg/ha)	Grain yield (%)
Week of the year				Hybrid rye L25P x L130N with imperfect male sterility					
17th BBCH 45	18th BBCH 47	19th BBCH 55	20th BBCH 59						
–	–	–	–	115 ± 4	2822 ± 410	74.7	25.3	24 ± 13	100.0
2	–	2	–	95 ± 5	34 ± 27	54.4	45.6	14 ± 10	1.2
2	2	2	2	89 ± 4	51 ± 8	29.2	70.8	8 ± 2	1.8
2	2	2	–	90 ± 5	19 ± 14	36.0	64.0	5 ± 3	0.7
–	2	–	2	105 ± 2	160 ± 14	53.6	46.4	64 ± 38	5.7
–	2	–	–	107 ± 3	187 ± 6	57.1	42.9	83 ± 16	6.6
–	2	2	–	105 ± 2	202 ± 59	49.9	50.1	102 ± 5	7.2
–	–	2	2	112 ± 2	642 ± 152	61.2	38.8	53 ± 2	22.7

[a] Measured at phenological stage BBCH 69.

Table 3. Effect of different MH application times on grain production and ergot yield for fertile rye variety Selgo and hybrid rye L25P x L130 N with imperfect male sterility infected by *C. purpurea* CCM 8405 (experiment run during the 2014 season).

The gametocide also did not affect the development of female gametophytes, as the ovaries remained susceptible to conidiospore infection and produced fully developed sclerotia (Fig. 4a). The ratio between harvested ergot and rye grain changed significantly (Fig. 4b), and ergot yield almost reached the value obtained using the hybrid male-sterile variety (Table 2).

Figure 4. Effect of MH on the infection progress and ergot yield in the experiment from the 2013 season. (a) *C. purpurea* infection on the fertile rye variety Selgo treated with three dosages of MH (2 kg per ha, left) and the non-treated plot (right). (b) Ergot harvest from the plot with the fertile rye variety Selgo treated with MH (3 x 2 kg per ha, left) in contrast to the harvest from non-treated plants (right). (c) Effect of four subsequent applications of 1 kg of MH per ha on the rye hybrid L25P x L130N (left) in comparison with the non-treated plot (right).

Previous studies have shown that sclerotia formed on a fertile rye are generally larger but fewer in number per ear (primary sclerotia) than are those on male sterile rye (mostly sclerotia from secondary infection), the former condition being considered a disadvantage for ergot harvest (Ne´meth, 1999). A different proportion of primary as opposed to that of secondary sclerotia on fertile versus sterile rye treated with MH was also observed in the 2013 season experiment (Table 2). This difference was not so obvious in the following year, however, thus suggesting that the size of sclerotia might be influenced also by weather conditions.

A lower dosage of MH (1–4 kg per ha), which already significantly decreased grain formation for the fertile rye variety, showed almost no effect on ergot yield. On the other hand, the time of first application seemed to be crucial for obtaining a good ergot yield with minimal grain contamination. Therefore, the field experiment in the 2014 season was directed mainly to finding the proper application time.

2.4.3. Application time of maleic hydrazide to maintain high yield and low grain/ergot ratio

Trials with varied times of gametocide application demonstrated the great importance of proper timing. Even the lowest dosage of MH (1 kg per ha) caused a dramatic reduction in the ergot yield on both those varieties used when it was applied earlier than at the beginning of heading, the stage when awns became visible (Table 2). Earlier application caused more distinctive changes than did later application of even three times the lowest dosage. Strong reduction in plant height, darker leaf color, and a delay in inflorescence emerging from flag leaf sheath (Fig. 4c) were observed in plants treated at a phenological stage between BBCH 47 and 49, thus indicating that processes other than pollen germination (e.g. female gametophyte development) might also be affected. The negative effect of earlier application was observed in two consecutive seasons. The positive effect of more than one application is also obvious from both seasons' experiments. Generally, the application of 2 kg per ha in 3 subsequent weeks before the field is in full flowering, but after the ear emerges from the leaf sheath, appears to be the most promising.

Lower ergot yield from the sterile variety in the 2014 season can be attributed to weather extremities. The spring of 2013 was extremely cold and wet with temperatures 0.8°C below and precipitation 116% above 30-year norms (1961–1990). On the other hand, the spring of 2014 had temperatures 2.4°C above and precipitation 86% below norms (Czech Hydrometeorological Institute; http://www.chmi.cz). In 2014, therefore, the development of rye plants was accelerated by approximately 2 weeks. As this estimation might not have been precise, the application of the first and second doses in 2014 was performed a couple of days earlier in relation to the plants' phenological stage. Moreover, fungal infections typically progress much better under cold and wet climate conditions (Ne´meth, 1999), which occurred during the spring of 2013 (in which year higher yields were obtained).

2.4.4. Inhibitory effect of maleic hydrazide on the germination of *C. purpurea* conidia

The inhibitory effect of MH applied in the form of the commercial preparation Fazor® on *C. purpurea* spore germination was tested *in vitro.* The results correspond well to the data obtained from the field experiments. Dosages of 1–3 kg of MH per ha showed only a weak negative effect on the ability of *C. purpurea* conidia to germinate. However, hyphae germinated from spores at MH concentrations above 3 kg per ha were significantly shortened. Nevertheless, if the time lag between infection and final gametocide application is not less than 5 days, the negative effect of the gametocide on spore germination can be ignored at a dosage around 2 kg per ha.

2.4.5. Ergot alkaloid content in sclerotia produced on rye treated with gametocides

Sclerotia grown on rye treated with MH and ETH during the 2014 experiment exhibited no significant alterations in EA content except for those that originated from plots with the earliest gametocide application.

2.5 Conclusion

Streamlining the ergot field production process can lead to a significant reduction in costs associated with manufacturing EA-based drugs. Male sterility that is chemically induced via an appropriate gametocide might minimize the impact of shortcomings in agro-technical practice. The procedure proposed in this work decreases the contents of organic contaminants coming from rye grain in the harvested sclerotia mass, but most importantly it permits the producer to use common fertile rye cultivars. The method of maintaining a population of fully sterile rye and preparing hybrid seed stock is challenging, and every failure by the human operator is followed by a large increase in undesirable grains contaminating the ergot. That means the costs of high-quality male-sterile hybrid seeds are as much as 20 times greater than are those of standard fertile rye cultivars. Plant protection products containing MH, an auxin antagonist with gametocide effect that was found to be the most promising, are readily available and relatively inexpensive. Thus, their application needs not significantly increase the costs of EA field production. Because *C. purpurea* is a living organism, sclerotia yield always varies from season to season, depending mainly on climatic conditions, as is also evident from the differing results obtained in the three subsequent seasons presented herein. Nevertheless, employing the gametocide can significantly increase the purity of the ergot while having little or no impact on its total yield if the application is accurately timed. Teva Czech Industries is presently one of the world's largest producers of EA-based drugs. The company's production is centered mainly on parasitic field cultivation and has generated undesirable losses over the past decade due to the heterogeneity of hybrid rye host plants. The first attempt at large-scale ergot cultivation on a fertile rye variety treated with gametocide brought a promising improvement in the cost-effectiveness of EA production for the 2014 season. Preliminary estimates of ergot yield range between 0.5 and 1 ton per hectare with organic impurities up to 30% for production at two independent locations (2 and 3 ha areas) using a fertile rye variety treated with two dosages of MH.

Part III – unpublished results - Introduction of an optimized method for the transformation of industrial strains *of Claviceps purpurea*

3.1 Abstract

An optimized method for the transformation of high-producing *C. purpurea* industrial strains was introduced in order to produce a large number of transformants per transformation. The method was used for the generation of two independent Gal 404 *LpsA2* knockout mutants and several overexpressors of ergot alkaloid synthesis (EAS) cluster genes.

In order to define the effect on the quantity of the alkaloids produced, three selected genes of the EAS were expressed constitutively under the control of a strong gpdA promoter in the Gal 404 strain and/or in Gal 130 strain, respectively. The verified transformants were inoculated on sterile rye plants and the sclerotia were harvested at two time points during infection, followed by alkaloid content analysis. HPLC analysis did not show any statistically important changes in the ergot alkaloid production of the transformants. The *LpsA1* gene from the ergotamine-producing 20.1 reference strain was constitutively expressed under the control of a PoliC promoter in Gal 130, an ergocristine-producing strain, resulting in significant changes in the relative representation of ergotamine in the EA pool in two independent transformants.

3.2 Introduction

A method of transformation producing a large number of transformants is essential "know-how" during the homologous recombination-based preparation of knockout mutants, because in *Claviceps purpurea*, homologous recombination occurs in 1-2% of the transformed cells (Haarmann et al., 2008).

The method of transformation from Mey et al., 2002, optimized in Hulvova, 2009, provides 1-2 transformants per transformation if the Gal 404 strain is transformed.

The introduction of a method optimized in order to produce a large number of transformants per transformation was a necessary step for the continuation of reverse genetic research focused on *C. purpurea*, industrial strains.

Because of their pharmaceutical usage, fungi-producing ergot alkaloids became a target group for improvement by genetic engineering methods. For a long time, random mutation and breeding were the main methods used for the improvement of industrial strains. Strains producing high levels of alkaloids, as well as strains producing alkaloids during submerged cultivation under specific conditions, were prepared in this way.

The discovery of the cluster of genes responsible for the production of ergot alkaloids, describing the function of all the genes that are present (reviewed in Gergards et al., 2014; Young et al., 2015) and genome sequencing of the main producers of ergot alkaloids (Schardl et al., 2013) opens up the possibility of targeted genetic manipulation.

Overexpression of the gene-encoding enzyme for the limiting step of the pathway determining the rate of secondary metabolite production can lead to the acceleration of secondary metabolite production.

Potential limiting steps could be catalysed by enzymes encoded by *dmaW*, *easC* and *easG* genes.

The *dmaW* gene, encoding enzyme catalyzing the first step of the biosynthetic pathway of ergot alkaloids, dimethylallyltryptophan synthase (Tudzynski et al., 1999).

The *easC* gene encodes catalase, which is involved in chanoclavine-I synthesis (Goetz et al., 2011). It was one of the two most up-regulated genes of the cluster, when the transcriptomes of alkaloid-producing structures – sclerotia - and low-productive structures – mycelia – were compared (Majeska et al., 2016).

EasG gene encodes enzyme catalyzing the conversion of chanoclavine-I aldehyde to agroclavine (Matuschek et al., 2011).

The *LpsA* and *LpsB* genes encode non-ribosomal peptide synthases (NRPS), responsible for the connection of D-lysergic acid with three amino acids, therefore ergopeptide biosynthesis. D-lysergic acid attached to LPSB is linked to bicyclic tripeptide, attached to LPSA in an amide-like fashion (Correia et al., 2003) or to LPSC-activated alanine (Ortel and Keller, 2009).

The *Claviceps purpurea* EA cluster consists of four NRPS genes: *LpsB, LpsC*, the ergonovine synthetase, responsible for alkylamide ergonovine production (Ortel and Keller, 2009), and two *LpsA* genes, encoding products, that are catalyzing the biosynthesis of ergopeptides (Haarmann et al., 2005).

The type of the first two amino acids present in tripeptide moiety determines the type of the final ergopeptide. The third amino acid is nearly always proline.

It was described in Riederer et al., 1996, by feeding studies of an isolated LPSA enzyme from the D1 strain of *C. purpurea*, that the substrate specificity of the enzyme is not absolute. Keller indicates that it is mainly the module responsible for position I that has broad substrate specificity and, like Riederer, mentions the dependence of the type of the final ergopeptide on the actual concentrations of the relevant amino acids in the cellular pool (Keller, 1999).

Nevertheless, on the evidence of knockout studies of *LpsA1* in the P1 strain, a new hypothesis was created, according to which absolute substrate specificity is expected, meaning that each LPSA enzyme catalyzes the formation of just one type of ergopeptide

(Haarmann et al., 2008). On the basis of this study, a number of recent publications (Lorenz et al., 2009; Gerhards et al., 2014) suggest the biotechnological improvement of industrially used strains by the knockout of one *LpsA* gene for single alkaloid production.

Very recently, the adaptation of a theory partially based on original hypotheses has been discussed. Transcriptome sequencing of the four industrial EA producers with different spectra of alkaloids produced showed significant differences in the expression of the genes involved in the biosynthesis of several amino acids, varying across strains with different spectra of alkaloids produced (Majeská et al., 2016).

Many well-described strains produce much higher numbers of types of ergopeptides than the number of *LpsA* genes that are present (there are usually two *LpsA* genes present in the strains described so far), even if they are produced in small amounts (see the description of the Gal 404 and Gal 130 strains in “Material and methods”).

Therefore, it is appropriate to modify the hypothesis and consider the expansion of the substrate specificity of the enzyme.

Heterologous expression of the *LpsA* gene (if the donor strain differs from the acceptor strain in the EA spectra produced) can contribute to the clarification of the substrate specificity of the enzyme.

3.3 Material and methods

The description of the transformation method is related to the Gal 404 strain, but identical procedure was applied to the Gal 130 strain.

3.3.1. Description of strains of microorganisms used in this study

C. purpurea, Gal 404 (patent CZ287130) and *C. purpurea*, Gal 130 (patent CZ279877) prepared by random mutagenesis and maintained by breeding were kindly provided by Teva Czech industries, s.r.o., Opava-Komárov, Czech Republic for research purposes.

The Gal 130 strain is one producing industrially high amounts of ergocristine. Its alkaloid spectra consist mainly of ergocristine, containing minor produced alkaloids: α ergokryptine, β ergokryptine, ergotamine, ergogaline, ergocornine and their stereoisomers –inins.

Gal 404 is a strong industrial ergotamine producer. Its alkaloid spectra consist mainly of ergotamine, containing minor produced alkaloids: ergotoxine, ergocristine, ergogaline, ergostin and their stereoisomers –inins.

The 20.1 strain (described in Schardl et al., 2013), used for the amplification of the *LpsA1* gene, the ergotamine and ergocryptine producer, was kindly provided by Prof. Tudzynski for research purposes.

Escherichia coli, TOP10 was purchased from Invitrogen, USA.

The FGSC 9721 strain of *Saccharomyces cereviceae* described in Winston et al., 1995

was kindly provided by Prof. Tudzynski.

3.3.2. Culture cultivation conditions

50 ml of liquid BII media (Esser et al., 1978) in a 250-ml Erlenmeyer flask was inoculated with Gal 404 strain culture (or the Gal 130 strain), growing on solid BII media. The liquid culture was cultivated for three days at 26 ˚C and 180 RPM.

Mantle agar (Mantle et al., 1976) was used as solid media inducing the sporulation of the Gal404 and Gal130 strains, needed for monosporic isolation and plant inoculation. The culture was grown for two weeks before the harvesting of the spores.

The yeast recombinational cloning was performed with the FGSC 9721 yeast strain (Winston et al., 1995) incubated at 30 °C in yeast extract-peptone-dextrose (YPD).

Standard cloning was performed with TOP10 strain of *E. coli bacteria*, incubated at 37 °C in LB media.

3.3.3. Vector description

pDrive plasmid (QIAGEN, Hilden, Germany).

GPDA_PROMO_pMK was commercially synthesized by Mr.GENE (http://mrgene.com).

The P434 and P444 vectors (Fungal Genetics Stock Center, http://www.fgsc.net/) were used for the preparation of hygromycin-resistant and phleomycin-resistant transformants by an optimized method for transformation. The P444 vector was used for the preparation of the vectors for the strong constitutive expression of the *dmaW*, *easC* and *easG* genes.

pRS426 (Colot et al., 2006) was used for the yeast recombinant cloning of the Gal404:Δ*LpsA2* vector.

The pNDH-OGG::lpsA1 gfp- vector was provided by Mgr. Michaela Hradilová; the preparation is described in Hradilová, 2016.

3.3.4. Molecular cloning

The primers were synthesized and delivered by Sigma-Aldrich, St. Louis, Missouri, USA). All the PCR reactions for the molecular cloning purposes were performed with proofreading, Phusion polymerase (NEW ENGLAND Biolabs, Ipswich, Massachusetts, USA). The PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich). The primers used for cloning the vectors are listed in Table 5.

The Gal 404:Δ*LpsA2* vector was constructed using the yeast recombinational cloning method. The regions covering the areas of the left flank and right flank were amplified using the 5F_lpsA2_GAL404, 5R_lpsA2_GAL404 and 3F_lpsA2_GAL404, 3R_lpsA2_GAL404 primers. The primers contain overlapping sequences towards the yeast shuttle-vector pRS426. The PCR products, the linearized pRS426 and phleomycin-resistant cassette

(amplified with the CpBleF1 and CpBleR1 primers from pRS426) were transformed into the FY834 yeast strain for homologous recombination.

The *easC* gene was amplified from the genomic DNA with the easCfull_fw and easCfull_rev primers, and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, and digested with EcoRI and ligated with EcoRI digested GPDA_PROMO_pMK (Hulvova, 2009), providing *easC*-GPDA_PROMO_pMK plasmid. The *easC*-GPDA_PROMO_pMK with *easC* integrated in positive direction was XbaI and Sac II digested and ligated with XbaI and Sac II digested p444, giving rise to a p444-*easC* vector, where the *easC* gene is localized between the gpdA promoter (the promoter for glyceraldehyde-3-phosphate dehydrogenace from *Aspergillus nidulans*) and the TrpC terminator from the same organism.The *easG* gene was amplified from the genomic DNA with the EasG_full_fw and EasG_full_rev primers and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, followed by XhoI, KpnI digestion and ligation with XhoI and KpnI digested GPDA_PROMO_pMK, providing *easG*-GPDA_PROMO_pMK. The e*asG*-GPDA_PROMO_pMK was SacII and XbaI digested and ligated with SacII and XbaI digested p444 plasmid (analogously to p444-*easC*), giving rise to a p444-*easG* vector.

The *dmaW* gene was amplified with the DMaWfull_fw and DMaWfull_rev primers and cloned into pDrive plasmid by a standard cloning technique, according to the manufacturer's recommendation, followed by EcoRI digestion and ligation with EcoRI digested GPDA_PROMO_pMK, providing *dmaW*-GPDA_PROMO_pMK. The *dmaW*-GPDA_PROMO_pMK was SacII and XbaI digested and ligated with SacII and XbaI digested p444 plasmid (analogously to p444-*easC* and p444-*easG*), giving rise to a p444-*dmaW* vector.

3.3.5. Transformation of *Claviceps purpurea*

The liquid culture in BII medium was centrifuged for 10 minutes at 5600g, RT and washed twice with SMaC buffer (0.85 M sorbitol, 0.2 M potassium maleate, 50 mM $CaCl_2$ pH 5.2, Mey et al., 2002).

The protoplast-generating solution was prepared by dissolving 5 mg/ml Lysing enzyme (*Trichoderma harzianum*, Sigma-Aldrich) in SMaC buffer, followed by sterilization by filter membranes with a pore size of 5 µm (Whatman® PTFE membrane filters, Sigma-Aldrich) and 0.22 µm (Millex syringe-driven filter unit, Sigma-Aldrich).

The cell precipitate was re-suspended in 20 ml of protoplast-generating solution and incubated for two hours at 28 ˚C, with shaking at 80 RPM.

Incubation was followed by filtration with a Nytex membrane (Franz Eckert, GmbH, Waldkirch, Germany), getting rid of the cell debris.

The protoplast suspension was centrifuged at 900 g, RT for 10 minutes and washed twice with STC buffer (0.85 M sorbitol, 10 mM Tris, 50 mM $CaCl_2$, pH 7.5, Mey et al., 2002).

The protoplast pellet was dissolved in 300 µl of STC buffer. The quality, purity and concentration of the protoplasts were determined under the microscope in a Bürker chamber.

The protoplasts obtained were transformed as follows:

10 µl of linearized DNA with a concentration of 1 µg/µl were added to 90 µl of STC buffer, followed by the addition of 50 µl of PEG solution (25% PEG 6000, 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.5, Mey et al., 2002) sterilized by filtration through a 5 µm-pore size filter membrane and 100 µl of protoplast solution. After 20 minutes of RT incubation, another 2 ml of PEG solution was added to the mixture and the transformation was finished after incubation lasting five minutes by the addition of 4 ml of STC.

625 µl of the cell solution after the transformation procedure was mixed with 20 ml of BII agar for regeneration (pH 8, no Fe^{2+} added, 200g/l sucrose).

One petri dish was antibiotic free, serving as a control of the vitality of the cells and ability to regenerate.

In the case of the preparation of phleomycin-resistant transformants (transformation performed with linearized p444 vector), the rest of the petri dishes were prepared by mixing cells with BII agar for regeneration containing 33 µg/ml of phleomycin, previously added directly to agar tempered at 50 ˚C.

When the hygromycin-resistant transformants were prepared (transformation performed with linearized p434 vector), after the transformation procedure the cells were mixed with antibiotic-free BII regeneration agar, followed by overlaying with 1.5 mg/ml of hygromycin containing BII regeneration agar after 12 hours.

The occurrence of antibiotic-resistant colonies was detected after 4-7 days of incubation

at 28 ˚C.

The colonies were re-inoculated on selection BII agar (pH8, no Fe^{2+}), containing 100 µg/ml of phleomycin or 500 µg/ml of hygromycin, respectively.

The growing colonies were used for DNA isolation and their parts were stored as water stocks at 4 °C.

3.3.6. DNA isolation and verification of transgene presence

DNA was isolated according to Cenis, 1992.

The presence of a transgene in the genome of transformants was verified by PCR analysis with the PHLEOres_fw, PHLEO res_rev and HYGROrt_fw, HYGROrt_rev primers listed in Table 5, and the number of integrated copies of the transgene by Southern blot analysis; the probes were prepared according to manufactures recommendation (DIG RNA Labeling Kit, Roche, Mannheim, Germany) from the *easC*-pDrive (*easc* gene was amplified with easCfull_fw, easCfull_rev primers) for p444-*easC* presence and copy number verification and from the phleo-pDrive (phleomycin resistance gene amplified with PHLEOres_fw, PHLEO res_rev primers) for p444-*easG* presence and copy number verification Primers are listed in Table 5.

The functionality of the vector to induce strong constitutive expression of the gene of interest was verified by Northern blot analysis (p444-*easG,* p444-*easC*), the probes were prepared with the EasG_full_fw, EasG_full_rev and easCfull_fw, easCfull_rev primers listed in Table 5, or by real-time PCR (p444-*dmaW*, Bražinová, 2012).

The location of the ∆*LpsA2* cassette was verified (homology recombination verification) with the lpsA2_fw, phleoHi3F2 (left flank integration) and lpsA2_rev, phleo_out_Hefe3 (right flank integration) primers listed in Table 5 and the absence of the wild-type allele was verified with the UTRza_lpsA1-gal404fw and lpsA2_k_zac_ rev primers listed in Table 5; see Figure 5.

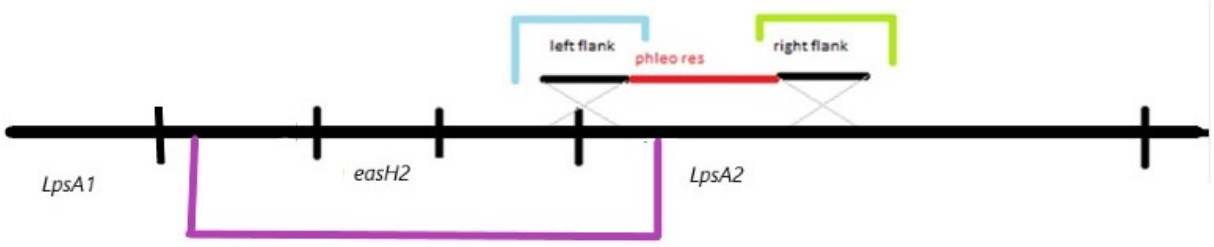

Figure 5. ∆*LpsA2* cassette. The blue line highlights the area amplified for verification of left flank integration; the green line highlights the area amplified for verification of right flank integration. The violet line highlights the area amplified for verification of the presence of the WT allele.

The presence of the OE*LpsA1*_20.1 cassette was verified by PCR, using the Hph fw and LpsA1-sek2_rev primers listed in Table 5.

Diagnostic PCR analyses were performed using the Dreamtaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by standard ethidium bromide-based electrophoreses in 1% agarose gel.

3.3.7. Southern and Northern blot analysis

The chemiluminescence-based method of detection was performed in Southern and Northern blot analysis.

Southern blot analysis was performed with 3-5 µg of EcoRI or ApaI digested, electrophoretically separated DNA in 1% agarose gel.

The separated DNA was transferred to a nylon membrane (Whatman Nytran nylon membrane, Sigma-Aldrich) and hybridized with a digoxigenin-labeled probe.

The isolation of the RNA was performed with an RNeasy mini kit (Qiagen). Turbo DNaseI (Thermo Fisher scientific, Waltham, Massachusetts, USA) treated RNA was separated on 1.2% agarose gel containing 0.4M formaldehyde. RNA was transferred to a nylon membrane (Whatman Nytran nylon membrane, Sigma-Aldrich) and hybridized with a digoxigenin-labeled probe.

3.3.8. Inoculation of sterile rye plants

Pollen sterile rye plants *Secale cereale* L. of a cultivar with a high affinity to *C. purpurea* infection (IHAR) were inoculated with transformants, as well as with an untransformed culture of the Gal 404 strain as a wild-type control. The inoculation of the plants was performed during the BBCH62 phenological stage, when approximately 30% of the anthers were mature, by rinsing ears with conidia suspension while puncturing them with a needle.

The infection proceeded in a greenhouse conditions at 20-25 °C/day and 15-18 °C/night.

The ears were covered by breathable fabric to avoid cross-infection.

The sclerotia were harvested and analyzed qualitatively as well as quantitatively for the production of ergot alkaloids by the HPLC technique.

3.3.9. Isolation of ergot alkaloids and HPLC analysis

The sclerotia were ground in a mortar and weighed and approximately 300 mg of the drug was extracted by means of slow shaking in a rotary shaker in 30 ml of extraction solution (0.3 ml of ammonium hydroxide and 29.7 ml of 90% acetone) for two hours. 10 ml of the extract were pipetted through glass wool and evaporated at 50 °C in a vacuum rotary evaporator. The sample was dissolved in 1 ml of 90% methanol and filtered through a 0.22-mm nylon filter and used for the HPLC analysis of the EA content on a Nexera system

(Shimadzu, Kyoto, Japan) with a C18 reverse-phase column (Zorbax RRHD Eclipse Plus, 1.8 mm, 2.1 mm ID x 50 mm, Agilent, Santa Clara, CA, USA).

0.001% (w/v) of each of the following alkaloids: ergotamine, ergostine, 8-hydroxyergotamine, ergocornine, α-ergokryptine, β-ergokryptine, ergocristine, ergogaline, ergotaminine, ergostinine, ergocorninine, α-ergokryptinine, β-ergokryptinine, ergogalinine and ergocristinine in 90% methanol (all from Teva Czech Industries, Opava-Komárov, Czech Republic) was used as the standard for the HPLC analysis. The analytes were eluted with solvents A (36 mM triethylamine/phosphate, pH 4.4, and acetonitrile, 4:1, v/v) and B (H_2O and acetonitrile, 1:4, v/v) using the following gradient: 0 min, 9% B; 10-12 min, 9-13% B; 12-14 min, 13-29% B; 14-16 min, 29-44% B; 18-20 min, 44-59% B; 26-28 min, 59-9% B, at a flow rate of 0.4 mL/min and a column temperature of 30°C. Monitoring was performed at 317 nm.

3.4 Results and discussion

3.4.1. Introduction of optimized method of transformation for Gal 404 and Gal 130 industrial strains

The optimized method introduced and described above provided about 100-200 colonies per transformation if applied to the Gal 404 and Gal 130 strains.

The introduction of a reliable method for the transformation of an organism of interest is essential “know-how” for reverse genetic research. In many research methods, such as recombinant homology-based knocking-out of a gene, it is necessary to screen large numbers of transformants to get the desired transformants and valuable data. An optimized method for the production of large numbers of transformants per transformation (i.e., up to 200 transformants per transformation) was introduced in the workplace of Department of Molecular Biology, Centre of region Haná, Palacký University with the kind help of Prof. Tudzynski’s group.

3.4.2. Generation of two independent Gal404:Δ*LpsA1* mutants

In order to describe the substrate specificity of the LPSA1 enzyme present in Gal 404, the ergotamine producer, two independent *LpsA2* knockout mutants were prepared. In total, 230 transformants were screened to obtain these two positive knock-outs meaning that efficiency of homologous recombination was 0,87%. The verified knockout mutants as well as the transformant with an ectopically inserted deletion cassette as a control, were inoculated on rye plants and the sclerotia thus formed were tested for changes in their production of ergot alkaloids.

Unfortunately, the analysis of the ergot alkaloids of the knockout sclerotia showed degeneration of the high-producing strain, resulting in a loss of the ability to produce ergot

alkaloids in the knockout mutants, as well as in the transformants with an ectopically integrated knockout cassette. The wild-type strain sclerotia which grew simultaneously produced a normal range of alkaloids typical of the strain being tested.

The degenerated culture had a reduced ability to infect plants. The production of honeydew was delayed by about two weeks, and the mass of the sclerotia decreased by up to 90%. The sclerotia differed from the wild type in their pigmentation and density, suggesting a loss of ability to develop mature sclerotia from the spacelial stage.

The degeneration of strains is a common problem complicating commercial production, as well as research and development of the production of ergot alkaloids (Chen et al., 2017). The combination of the necessary maintenance breeding and the process of transformation is difficult and it is always necessary to consider this option during the data interpretation.

Nevertheless, with the example of the Gal 404:Δ*LpsA2* mutant, we demonstrated that all the necessary steps for the preparation of the knockout mutants of the high-producing strain Gal 404 were introduced and can be used in future for preparation of knock-out strain of any kind of gene from *Claviceps* genome.

3.4.3. Strong constitutive expression of three genes of the EA cluster

Three genes of the ergot alkaloid cluster (ie. *dmaW, easC* and *easG*) were constitutively expressed under the control of a strong gpdA promoter in two strains producing large amounts of ergot alkaloids. Diagnostic PCR verified the presence of a transformation cassette, while Southern blot analysis verified multiple integration of a transgene in most of the tested transformants. Constitutive expression of a transgene was verified by Northern blot analysis (Gal 404:OE*easG*, Gal 404:OE*easC*) or by real-time PCR (Gal 130:OE*dmaW*, Bražinová, 2012) with RNA isolated from mycelia, where the genes of the EA cluster are not expressed or show very low expression. The obtained data suggest that none of genes selected for overexpression in industrial strains is a significant limiting factor for the production of ergot alkaloids in these infectious, strongly ergot alkaloid-producing strains. Although the limiting factors may vary in different strains, especially among strains producing alkaloids in submerged culture and infectious strains.

3.4.4. Heterologous constitutive expression of *LpsA1* gene in Gal 130

The *LpsA1* gene, responsible for the production of ergotamine in the 20.1 strain (Haarmann et al., 2008), was constitutively expressed in the Gal 130 strain, the ergocristine producer. The transformation was verified by diagnostic PCR.

Two independent transformants were inoculated on rye plants and sclerotia harvested at three time points were analyzed for changes in their ergot alkaloid spectra (see Table 4.).

19 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	91.0	94.9	95.8
	αErgokryptine+inine	2.6	0.0	3.1
	Ergotamine+inine	6.4	5.1	1.1
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	90.3	95.8	98.1
	αErgokryptine+inine	2.7	0.0	1.7
	Ergotamine+inine	7.0	4.2	0.2
	3. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	91.7	89.1	96.0
	αErgokryptine+inine	2.4	1.9	3.0
	Ergotamine+inine	5.9	9.0	1.0
31 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.3	88.4	96.0
	αErgokryptine+inine	2.9	4.5	3.0
	Ergotamine+inine	2.8	7.1	1.0
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.2	94.5	96.6
	αErgokryptine+inine	2.9	2.6	2.7
	Ergotamine+inine	2.9	2.9	0.6
55 dpi	**1. Biological replicate**	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	93.0	91.0	95.0
	αErgokryptine+inine	4.1	0.0	3.5
	Ergotamine+inine	2.9	9.0	1.4
	2. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	93.8	90.3	94.8
	αErgokryptine+inine	3.6	2.9	3.9
	Ergotamine+inine	2.6	6.7	1.3
	3. Biological replicate	**Gal130:OE*LpsA1* 11 (% from total EA)**	**Gal130:OE*LpsA1* 6 (% from total EA)**	**Gal 130 WT (% from total EA)**
	Ergocristine+inine	94.8	71.4	95.6
	αErgokryptine+inine	3.0	0.0	3.1
	Ergotamine+inine	2.2	28.6	1.2

Table 4. Changes in relative ergotamine representation in EA pool of two independent transformants Gal 130:OE*LpsA*1,20.1 compared to Gal 130 WT.

Significant qualitative changes in the EA spectra of two independent Gal 130:OE*LpsA1*, 20.1 transformants were monitored at three time points during infection. The effect of heterologically expressed *LpsA1*, and therefore changes in ergotamine representation, were significant mainly in the early stages of the development of the sclerotia, when the expression of naturally occurring ergot alkaloid cluster genes is lower. Affecting the ergot alkaloid spectra by heterologous *LpsA* expression could open new possibilities for the designing of new *C. purpurea* production strains, although the size of the gene (i.e., approx. 11 kb) can delimitate the level of expression.

Using a suitable, strong promoter, the heterologous expression of *LpsA* can lead, e.g., to the preparation of submerged producing strains with altered EA spectra, although the supposed effect of the actual concentration of the relevant amino acid on the type of produced EA in the strain must be considered.

3.5 Conclusion

A method for *C. purpurea* transformation providing a large number of transformants per transformation was successfully introduced.

The method was used for the preparation of two independent Gal404:Δ*LpsA2* mutants in order to determine the substrate specificity of the LPSA1 and LPSA2 enzymes by monitoring changes in the EA spectra and focusing on both the major and minor alkaloids that are produced. Strain degeneration resulting in total loss of EA production made evaluation of the experiment impossible.

Three genes of the ergot alkaloid pathway, i.e., *dmaW*, *easC* and *easG*, were constitutively expressed in the Gal 404 and/or Gal 130 strains under the control of a strong gpdA promoter. The mutants were screened for changes in their total production of alkaloids. No biotechnologically significant, sustainable changes in EA production were observed.

The *LpsA1* gene originating from the 20.1 strain, the ergotamine producer, was constitutively expressed in the Gal 130 strain, the ergocristine producer. Two independent Gal 130:OE*LpsA1*, 20.1 mutants were monitored for changes in ergotamine representation in the EA pool at three time points during infection. A significant improvement in ergotamine representation up to 28% of total EA content, which is originally the minor alkaloid of the strain, representing in WT only around 1% of the EA pool, was observed in both mutants.

Primer name	Sequence (5´-3´)
EasG_full_fw	CCGGAATTCATGACGGTCTTACTGACAGGAGG
EasG_full_rev	CGGGGTACCTCACTTTCTTGCACGCCAG
easCfull_fw	ATGGCTTCTGAGGTCTCTG
easCfull_rev	TTACTCCACTATCTCTTCGG
DMaWfull_fw	ATGTCGACCGCAAAGGACCCAG
DMaWfull_rev	CTACTTYGTKGAGAGKTCACAGCG
PHLEOres_fw	GACTCCGTGGAGGACGACTT
PHLEOres_rev	TGCTCGCCGATCTCGGTCAT
HYGROrt_fw	CGAGGTCGCCAACATCTTCT
HYGROrt_rev	GCGTCTGCTGCTCCATACAA
UTRza_lpsA1-gal404fw	AGGCCGATACTGCGTTAC
lpsA2_k_zac_rev2	TTCCGAGAACTACAGCCCA
Hph fw	CCTCCACTAGCTCCAGCCAAGCCC
LpsA1-sek2_rev	AGCTTCACAACAAATGGCGT
5F_lpsA2_GAL404	CCAGGGTTTTCCCAGTCACGACGGGATCCCTGGACGGGCAATCAATCAG
5R_lpsA2_GAL404	CCACTTAACGTTACTGAAATCTCCAACCAGCAGAGCCGGACGATATA
3F_lpsA2_GAL404	CTCCTTCAATATCATCTTCTGTCTCCGACTGTGGTCAACGCCGAGAGTA
3R_lpsA2_GAL404	ACAATTTCACACAGGAAACAGCGGATCCTGGAGAGTCTGGGCATCTAAC
dia_lpsA2_GAL404_fw	CACCGACAAGTTGTGGACAC
dia_lpsA2_GAL404_rev	TAATAACTGGAGGCCCTATAG
phleo_Hi3F2	GTGTTCAGGATCTCGATAAGATACG
Phleo_out_Hefe3	GAGCTCGGTATAAGCTCTCC
CpBleF1	CGGAGACAGAAGATGATATTGAAGGAGCGATCGAGACCTAATACAGCCCC
CpBleR1	GTTGGAGATTTCAGTAACGTTAAGTGGGCATTGCAGATGAGCTGTATCTG

Table 5. Oligonucleotide primers used in the study

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