BSL 3: Alternative strategies to by-pass the plant-based Azadirachtin-A production

Peter Spieth[1], Rieke Lohse[1], Hanna Bednarz[2], Hubertus Kleeberg[3], Karsten Niehaus[2], Anant V. Patel[1]

[1]University of Applied Sciences Bielefeld. Interaktion 1, D-33619 Bielefeld, Germany, e-mail:peterspieth@fh-bielefeld.de
[2] Bielefeld University, Universitätsstraße 25, D-33615 Bielefeld, Germany
[3]Trifolio-M GmbH, Dr.-Hans-Wilhelmi-Weg 1, D-35633 Lahnau, Germany

DOI 10.5073/jka.2016.453.009

Abstract

All parts of Neem (*Azadirachta indica* A. Juss) show a broad spectrum efficacy against insect pests including insecticidal, anti-feedant or insect repellent activities. Several studies have shown that plant cell cultures can produce azadirachtins. We induced more than 40 novel Neem cell lines in modified Murashige and Skoog (MS) media containing different concentrations of auxins and cytokinins. To enhance the Azadirachtin production from 1 mg/l, it was necessary to optimize the media composition separately for biomass and secondary metabolite production. In light of this complex challenge we used our novel fully automated high-throughput microbioreactor system that allows us a fast and controlled batch and fedbatch screening in 48-well microtiter plates. There is increasing evidence that plants like *Azadirachta indica* contain endophytes which are able to colonize internal plant tissue without causing visible disease symptoms. The estimated high species diversity of endophytes suggests a rich and almost untapped source of new secondary metabolites. We isolated more than 340 endophytes from various plant tissues and tested if they were able to produce Azadirachtin-A. Here, we present data on isolation of endophytes and induction of callus as well as first results of our microbioreactor system.

Keywords: Azadirchtin-A, endophytes, plant cell cultures, high-throughput microbioreactor

Introduction

The neem tree belongs to the Meliaceae family, it originated from Asia and is currently grown in all subtropical areas around the world (Rodrigues et al., 2014). Its use for therapeutical and agrochemical applications has enhanced its industrial value. The tree produces various secondary metabolites with different biological effects for commercial applications. The best known active compound is Azadirachtin-A, which is already used as broad-spectrum biopesticide. Common ways for the extraction of the active compounds are still expensive and very time consuming. In consideration of the fact that the interest on plant secondary metabolites for the pharmaceutical and agricultural application has increased rapidly in the last years new and complementary ways must be found to satisfy the demand of these active compounds. The challenge during the development of new production strategies is to preserve the production potential of the strain or culture. A multiplicity of studies have shown that the use of submersed plant cell cultures is a promising way to create a competitive production procedure compared to conventional manufacturing processes. Besides the plant cell cultures, endophytes seem to be involved or even responsible for the production of pharmaceutical compounds, such as Taxol (Stierle et al., 1993) and Camptothecin (Rehman et al., 2008) as well as Azadirachtin-A (Kusari et al., 2012)

The overall aim of a BMBF funded project is to develop a competitive process to produce high concentrations of bioinsecticidal compounds with Neem plant cell cultures or endophytes. The bioactivity of the produced compound is tested in a high-throughput bioassay based on Sf9 cells.

Materials and Methods

Induction of plant cell lines and isolation of endophytes

In a first step various plant tissues were surface sterilized as previously described (PRAKASH and SRIVASTAVA, 2008). In case of the isolation of endophytes the protocol was optimized related to the time and concentration of the used chemicals. Afterwards cut explants were incubated on appropriate media followed by a transfer into submersed cultivation in shake flasks.

Extraction and detection of secondary metabolites

The secondary metabolites of the submersed cultures were extracted using a commonly applied liquid liquid protocol as well as following a novel *in situ* product removal strategy based on resin beads. The received samples were analyzed via UHPLC-DAD-ESI-TOF-MS/MS measurement.

Results

We could successfully induce callus cultures from stem and leaf tissues. For this we analyzed more than 4200 explants incubated in the dark as well as with a dark/light rhythm of 8 to 16 hours. For example, figure one shows from how many stem explants we could finally induce callus material. Not surprisingly the MS media containing synthetic cytokinins and auxins showed the highest callus proliferation from more than 90 % of the explants (Figure 1).

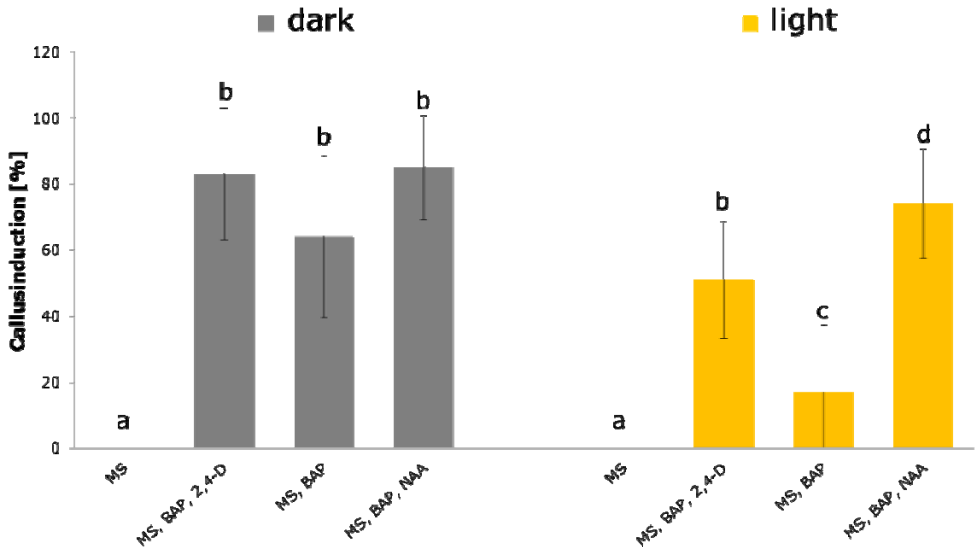

Fig. 1 Influence of medium compositions on callus induction mean (±SD) of stems; n=20. Different letters above bars indicate significant differences according to Kruskal-Wallis test with Mann-Whitney-U post hoc test at $P<0.05$.

The isolation of endophytes from different tissues and locations has led to 346 isolates so far (Figure 2). Most endophytes were isolated from leaf and stem tissues, 56 % of them were fungi and 44 % were bacteria.

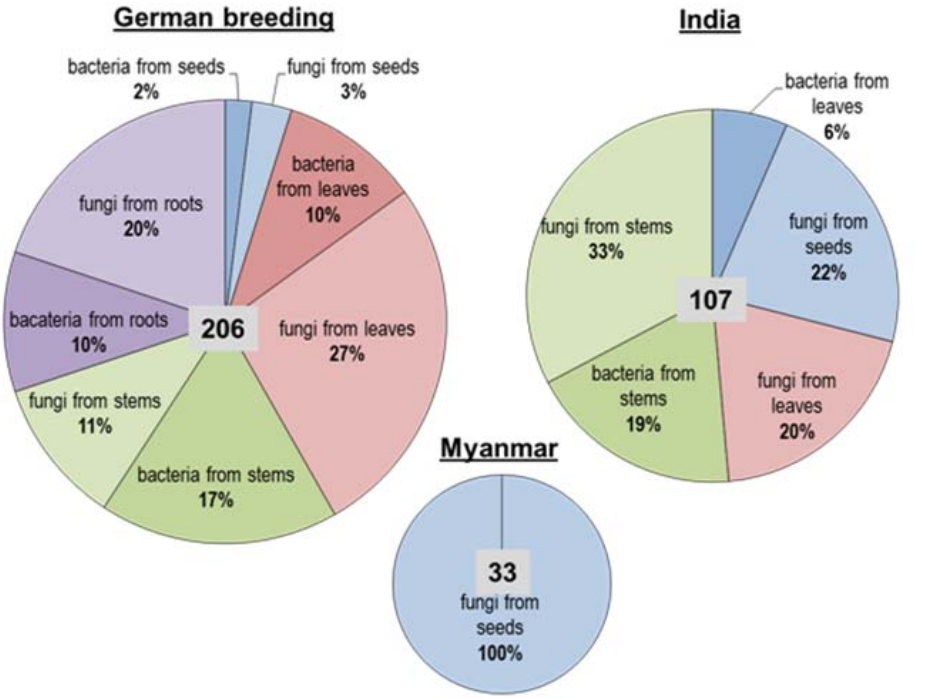

Fig. 2 Total number of isolated endophytes from various plant tissues and destinations.

The measurement via UHPLC-DAD-ESI-TOF-MS/MS allows a specific identification of Azadirachtin-A. We were able to detect the expected mass fragments of Azadirachtin-A in 25 % of our plant cell lines (Figure 3).

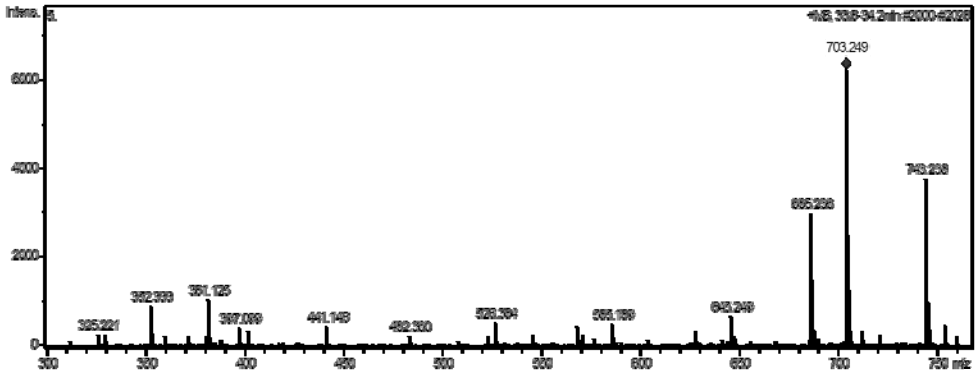

Fig 3: Mass spectrometry data of extracted Azadirachtin-A from plant cells including the expected fragment masses.

In on-going experiments we use the microbioreactor system to optimize the media composition to increase the Azadirachtin production. Furthermore we use the microbioreactor to develop an automated high-throughput screening, to test the efficacy of endophytic isolates against Sf9 cells.

References

KUSARI S, VERMA VC, LAMSHOEFT M AND SPITELLER M. 2012. An endophytic fungus from Azadirachta indica A. Juss. that produces azadirachtin. World J Microbiol Biotechnol 28: 1287-1294.

REHMAN S, SHAWL AS, KOUR A, ANDRABI R, SUDAN P, SULTAN P, VERMA V AND QAZI GN. 2008. An endophytic Neurospora sp. from Nothapodytes foetida producing camptothecin. Appl Biochem Microbiol 44: 203-209.

RODRIGUES M, FESTUCCI-BUSELLI RA, SILVA LC AND OTONI WC. 2014. Azadirachtin biosynthesis induction in Azadirachta indica A. Juss cotyledonary calli with elicitor agents. Brazilian Archives of Biology and Technology 57: 155-162.

STIERLE A, STROBEL G AND STIERLE D. 1993. Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Science 260: 214-216.