Host recognition of *Orobanche cumana,* the broomrape of cultivated sunflower

Otmar Spring, Frank Raupp
Institute of Botany, University of Hohenheim, D-70593 Stuttgart, Germany, o.spring@uni-hohenheim.de

ABSTRACT

- **Background and aims:** Most of the economically relevant parasitic weeds of the Orobanchaceae have a more or less broad host spectrum within the plant family they are adapted to. The host-released compounds inducing their germination were identified as so-called strigolactones and represent four-ring terpenoids derived from the carotenoid metabolism. *O. cumana*, a fast emerging problem of sunflower cultivation in areas with dry and hot summers, similarly reacts on strigolactones (e.g. GR24), but accepts no other host than *Helianthus annuus*. However, from sunflower no strigolactone was described yet. Previous reports indicated, that sesquiterpene lactones (STL) may trigger a similar reaction in *O. cumana* and recently dehydrocostus lactone (DCL) was identified as a first natural germination inductor of this type from sunflower root extracts. However, studies on *H. annuus* / *O. cumana* interaction showed that more than one inducing compound is released from sunflower root exudate. We aimed to identify their chemical nature and specificity.
- **Methodology:** Bioassay-guided HPLC purification of root exudate compounds. MS and NMR spectroscopic analysis for structure elucidation. Comparative germination tests.
- **Key results:** HPLC separations of root exudates from hydroponic sunflower cultivation indicated several fractions with stimulating activity for *O. cumana* seed germination. Besides the pseudoguainolide DCL, we identified the germacranolide costunolide and the xanthanolide tomentosin as bioactive compounds. All three STL are new for *H. annuus* and were not found in STL-producing glandular trichomes of leaves and flowers. The STL induced germination of *O. cumana* at an ED_{50} of ca. 1-7 nM. Concentrations above 1 µM reduced their activity and overdose application inhibited germination irreversibly. This is most likely a consequence of the nucleophilic reactivity known from many cytotoxic effects of STL. In addition, STL from glandular trichomes of aerial parts of sunflower caused similar stimulating effects. The content of less than 1 trichome was sufficient to induce germination of seeds. Simple leave washings with water, simulating natural rain events, strongly induced germination as well. While *O. cumana* proved higly sensitive for STL from roots or leave leachates of its host, no such effects of STL were found with *Phelipanche ramosa* in our tests.
- **Conclusions:** *O. cumana*, unlike other species of the genus reacts on STL signals of its host. At the same time, sunflower broomrape shares with its relatives a similar sensititvity for strigolactones. This indicates a structural alteration in the receptor, necessary for recognition of this new type of germination stimulant. For *O. cumana* this may have been an evolutionary key step for the host jump to Asteraceae which seemingly lack the production of typical strigolactones.
- **Contribution to the current knowledge:** STL from sunflower were identified as key elements in the interaction with *O. cumana*. This allows development of new strategies to control this economically important parasitic weed.

Key words: Broomrape - *Helianthus* – *Orobanche* – sunflower – strigolactone - sesquiterpene

INTRODUCTION

Parasitic weeds of the genera *Striga*,. *Orobanche* and *Phelipanche* are a serious problem in many crops, particularly in Poaceae, Fabaceae and Solanaceae. Germinating from tiny seeds, a common feature of these parasites is their instant necessity to attach to their host's root system, to penetrate the central cylinder and to attach to the vessels in order to get access to water and nutrition. While *Striga* species usually have a narrow host range within the Poaceae, members of the two other genera often accept a broader variety of host species within the dicots. The host specificity is regulated by two factors: 1. the resistance of host plants against the formation of parasitic haustoria; 2. the release of chemical signals that activate germination of parasitic seed in only millimeter distance of the host root. Strigol, a four-ring terponoid from the carotenoid metabolism was the first germination activator identified (Cook et al., 1966). Since then, numerous similar derivatives of so-called strigolactones have been identified and functionally analysed so that the active structural part was identified and implemented in a synthetic chemical activator, namely GR24 (Thuring et al., 1997).

Gr24 was shown to induce germination of *Striga*, *Phelipanche* and *Orobanche* species. This includes *O. cumana*, the sunflower broomrape (Fig. 1), a parasite of fast growing economic importance in many countries around the world (Parker, 2009). The natural signal of sunflower for the stimulation of seed germination in *O. cumana* remained unknown until recently Joel et al. (2011) identified the sesquiterpene lactone (STL) dehydrocostuslactone (DCL) as a responsible compound in sunflower roots. The capacity of STL to stimulate seed germination of parasitic weeds was known long before (Fischer et al., 1989) and sunflower leaves are a rich source for theses compounds (Spring and Schilling 1989), but neither had DCL been isolated from *Helianthus annuus* before, nor had any other STL been isolated from in the roots of this taxon. Our chemical analysis of sunflower root exudates confirmed the presence of DCL, but at the same time indicated that other germination stimulants are released as well and need to be identified.

Fig. 1.: *Orobanche cumana* in a field of oilcrop sunflower

MATERIALS AND METHODS

Seeds of *O. cumana* were surface sterilized with hypochloride (1%, 5 min) and kept for one week in darkness at 16° C on wet filter paper (Whatman filter type GF/A) before use in germination bioassays. This method of preconditioning has been reported to improve the ratio and speed of germination (Magnus et al. 1992). To test the site of stimulant release, six days old sunflower seedlings were placed horizontally on water agar in a petri dish. Stripes of filter paper with seeds of *O. cumana* were placed on the plant surface for 24h and then stored for six days in darkness on wet filter paper, before the ratio of germinated seeds was counted under a stereo microscope. Roots of sunflower seedlings (surface sterilized before germination and cultivated on wet filter paper) were extracted with acetone. The extract was dried, suspended in CHCl3 and partitioned against water. The chloroform fraction was dried, suspended in a mixture of pentane, methanol and water (3:3:1) and partitioned again. The methanol-water fraction was

used for preparative HPLC and further purification of compounds. All steps from the crude extract to the chromatographic fractionation were monitored with a bioassay for stimulation of *O. cumana* germination as described above. GR24 (10^{-6}M) was used as control. Specificity of the bioactivity was tested against seeds of *Phelipanche ramosa*, the broomrape of tobacco.

To test the natural release of germination stimulants from roots, sunflower seedlings were raised on wet filter paper for ca. five days and then transferred to hydroponic cultivation in tap water (Fig. 2). The hydroponic solution was pumped over a column with Amberlite XAD resin (Sigma, Taufkirchen, Germany). Every 24h, the water was replaced and the absorbed compounds were washed from the column with acetone. The exudate samples were dried, dissolved in MeOH and tested with the bioassay for stimulating activity as described before.

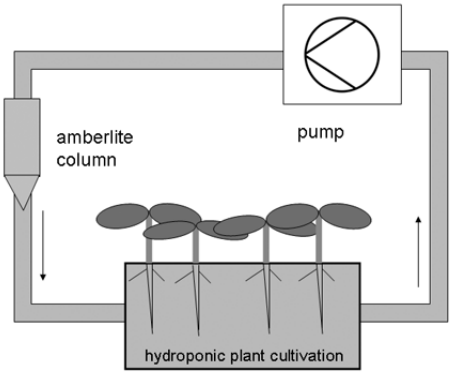

Fig. 2. Scheme for sampling sunflower root exudate by means of cycling the hydroponic solution over a column with amberlite resin in order to absorbe organic compounds released from the root.

The activity of STL from glandular trichomes of sunflower leaves was assayed by mechanical sampling of trichomes with a fine needle and extracting them with MeOH as described previously (Spring, 1991). Additionally, the natural activity of aerial plant parts towards *O. cumana* was investigated using leaf leachates. Fresh leaves of a ca. 5 week old sunflower were sprayed with deionized water and the leachate was collected with a funnel at the tip of the leaf. The samples were tried *in vacuo*, the residue was dissolved in MeOH and tested in the bioassay as described above.

RESULTS AND DISCUSSION

Seeds of *O. cumana*, placed with wet filter paper on the surface of sunflower seedlings readily germinated when they had been in contact with the rhizodermis and with the meristematic root zone (Fig 3). A reduced germination activity was found in old root zones and contact to the hypocotyl and cotyledone surface had hardly any effect. This indicates that stimulating compounds are naturally released from the roots of sunflower, where the tissue of the plant is not or only little protected with a cuticle that inhibits exchange with the hydrophilic environment.

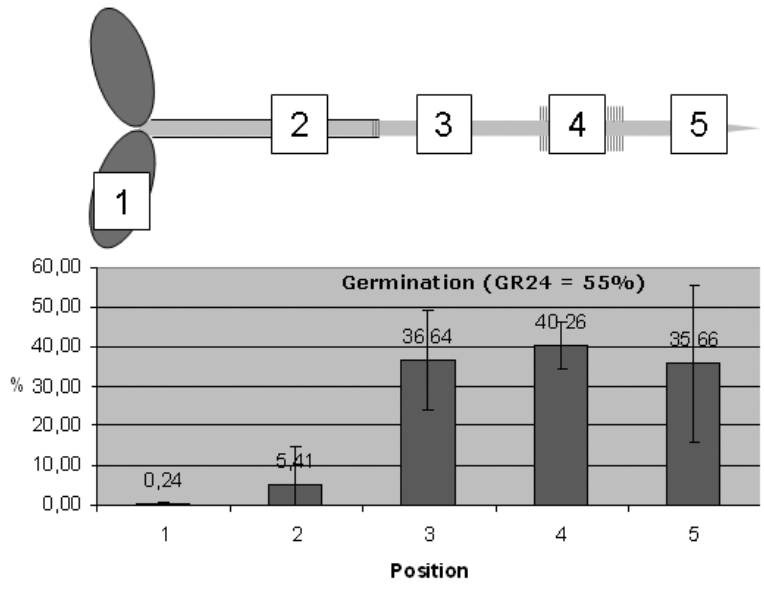

Fig. 3. Bioassay for locating the site of release of germination stimulants. Seeds (ca. 150 per sample) of *O. cumana* on wet filter paper were placed on the surface of sunflower seedlings for 24h at the positions given in the scheme: 1, cotyledone; 2, hypocotyl; 3, older root part; 4, hairy root zone; 5, root tip. Seed germination was investigated six days later and compared with samples treated with GR24 (10^{-6} M).

Guided by this observation, root exudates were collected from hydroponic cultures of sunflower seedlings and tested for bioactivity. The exudates showed strong, but sometimes highly variable activity in comparison to controls tested with GR24. Surprisingly, in samples with weak activity the effects could rarely be improved when higher concentrations were applied, but often a 1:10 or 1:100 dilution raised the ratio of germination to the level of GR24 or higher. This indicated that the stimulating activity could be overcome by inhibiting effects and hampered attempts to purify the responsible compounds by bioassay-guided fractionation.
HPLC analysis of the root exudate on RP18 material revealed a broad array of hundreds of hydrophilic to hydrophobic compounds (Fig. 4). Among them, a peak with the retention time and UV spectrum of DCL was identified in the hydrophobic zone of the diagram. Its MS and ^{1}H NMR spectroscopic data confirmed the structure of DCL (Hikino et al., 1964; Yuuya et al., 1999). This corroborates with the results of Joel et al. (2011) who extracted DCL from the roots and identified its presence in exudates indirectly by spiking the suspicious HPLC peak with a reference sample.

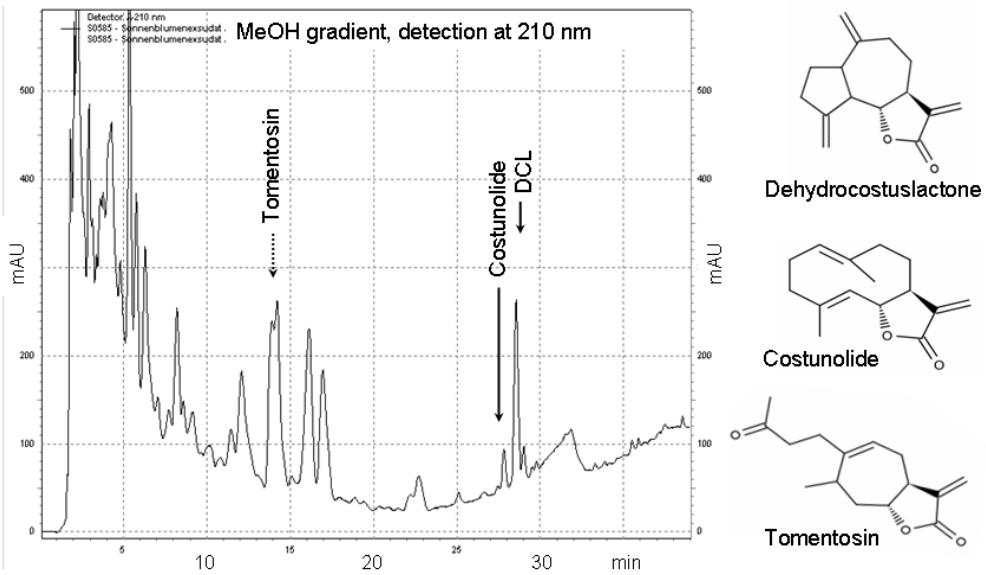

Fig. 4. HPLC diagram (at 210 nm detection) of a sunflower exudate separated on a RP18 column in a methanol/water gradient. Bioactive fractions were purified for spectroscopic measurements. Purification of tomentosin necessitated a subfractionation in acetonitril/water.

Bioassays of the HPLC fractions unquestionably made clear, that DCL is not the unique germination stimulant of sunflower for *O. cumana*. Next to DCL, a second active compound was found and spectroscopically analyzed. Its spectral data were identical with costunolide (Yabuta et al., 1978), the first and most simple STL in the biosynthesis of these compounds. Its formation via C-6 hydroxylation of germacrene acid has recently been shown employing heterologous enzymes in a multi-vector yeast transformant (Ikezawa et al. 2011). Although predictable as a precursor of the STL found in sunflower glandular trichomes, costunolide has now been identified for the first time in *H. annuus*.
Moreover, in the more polar part of the HPLC diagram, a third bioactive compound with UV characteristics of STL occurred. Its MS and ^{1}HNMR spectroscopic undoubtedly identified it as tromentosin (Bohlmann et al., 1978). This compound belongs to the rare skeletal type of xanthanolides. The compound has not yet been found in any species of the genus *Helianthus*, but it occurs in trichomes of the related *Pappobolus* and *Scalesia* spp. (Spring et al. 1991b; 1997). Obviously, the formation of STL

in glandular trichomes of aerial parts is different and independent from the metabolites produced in the roots.

From the previous tests it was not surprising that we found similar bioactivity with STL from the glandular trichomes of sunflower. The methanolic extract of a single trichome, mechanically collected from the leaf surface was sufficient to induce germination in ca. 20% of the seeds. Although STL of the glandular trichomes are usually sequestered into a globe shaped structure at the trichome tip were they are covered by cuticular layer, we were interested to see whether they might naturally be washed out by rain and could reach the soil in sufficient amounts to stimulate seed germination of *O. cumana*. Leaf leachates from gently spraying water on the surface of sunflower leaves induced germination (the leachate from 0.1 cm^2 induced 75 % germination.).

Bioassays with the purified STL showed similar quantitative activities. In all cases, the stimulation of germination started at low nM concentration and reached a maximum at around 0.1 to 1 µM (Fig. 5). Above this level, the ratio of germination decreased rapidly towards 0 at 10^{-4}M. This expands the observations of Joel et al. (2011) who tested DCL up to the µM range where the maximum activity was found. The inhibition caused by an overdose of STL might be explained by cytotoxic effects which are exaggerated by the exocyclic methylene group at the lactone ring. This group is known to react easily with nucleophiles. On the other hand this functional group seems to be essential for the stimulation of *Orobanche*, as it has been shown by Zwanenburg et al. (2009) for strigolactones and by Joel et al. (2011) for DCL. In our experiments, the absence of germination after treatment of *O. cumana* seeds with 10^{-4}M costunolide could not be compensated by subsequent treatment with GR24. The germination seemed to be blocked irreversibly.

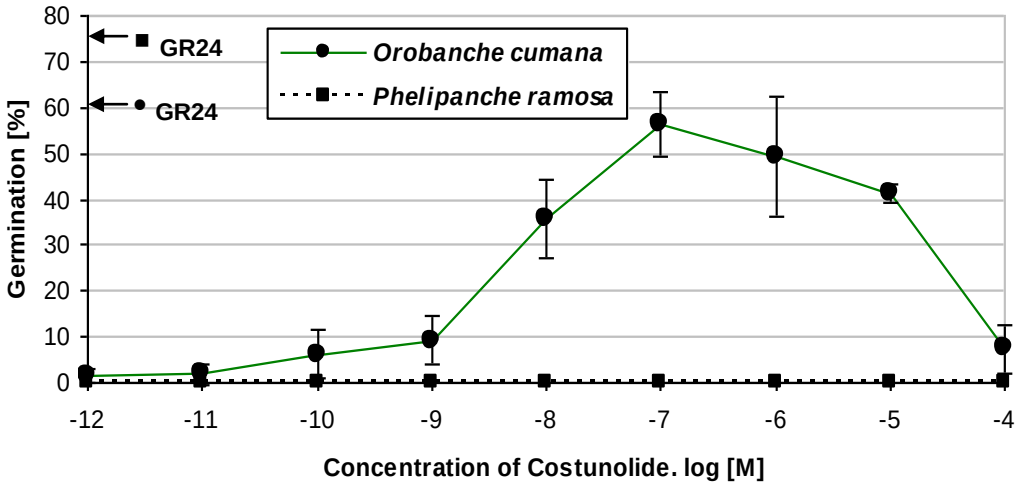

Fig. 5. Dose response of *O. cumana* and *P. ramosa* to costunolide. GR 24 (10^{-6}M) was used as control.

With DCL (10^{-6}M), Joel at al. (2011) observed a more than 50% stimulation of the germination of *Phelipanche aegyptiaca*, although this species has its natural host range in the Solanaceae, from which only strigolactones are known to be released in root exudates (Xie et al., 2007). A similar experiments with costunolide did not induce any germination in *P. ramosa*, the broomrape of tobacco (Fig.5). This was surprising to us and requires additional experiments in the future with respect to the specificity of STL towards other parasitic weeds. We have not yet found any other *Orobanche/Phelipanche* species to react on STL in a similar sensitivity as *O. cumana*. At the same time, sunflower broomrape shares with its relatives the same sensititvity for strigolactones. This was not only observed with GR24, but also with experiments involving other crop plants (e.g. tobacco, barley etc.) which induced seed germination of *O. cumana*, but did not allow attachment to their roots. From the evolutionary point of view this could be indicative for differences in the receptor of Orobanchaceae taxa and might be a key factor for speciation. For agriculture this could help to control sunflower broomrape with appropriate crop rotation.

ACKNOWLEDGEMENTS
We gratefully acknowledge excellent support in spectroscopic measurements by J. Conrad, Institute of Bioorganic Chemistry, University of Hohenheim as well as technical assistance by Patrick Mack and Sven Reichardt.

REFERENCES

Bohlmann, F., P.K. Mahanta, J. Jakupovic, R.C. Rastogi, and A.A. Natu. 1978. New sesquiterpene lactones from *Inula* species. Phytochemistry 17:1165-1172.

Cook, C. E.; L.P. Whichard, B. Turner, M.E. Wall, and G.H. Egley. 1966. Germination of Witchweed (*Striga lutea* Lour.): Isolation and Properties of a Potent Stimulant. Science 154:1189-1190.

Fischer, N.H., J.D. Weidenhamer, and J.M. Bradow. 1989. Dihydroparthenolide and other sesquiterpene lactones stimulate witchweed germination. Phytochemistry 28:2315-2317.

Hikino, H., K. Meguro, G. Kusano, and T. Takemoto. 1964. Structure of mokkolactone and dehydrocostus lactone. Chem. Pharm. Bull. 12:623-634.

Ikezawa, N., J.C. Göpfert, N. T. Nguyen , S-U. Kim, P.E. O'Maille, O. Spring, and D.-K. Ro. 2011. Lettuce Costunolide Synthase (*CYP71BL2*) and its homolog (*CYP71BL1*) from sunflower catalyze distinct regio- and stereoselective hydroxylations in sesquiterpene lactone metabolism. J. Biol Chem. 286:21601-21611.

Joel, D.M., S.K. Chaudhuri, D. Plakhine, H. Ziadna, and J.C. Steffens. 2011. Dehydrocostus lacotne is exuded from sunflower roots and stimulates germination of the root parasite *Orobanche cumana*. Phytochemistry 72:624–634.

Magnus, E., P. Stommen, and B. Zwanenburg. 1992. A statndardized bioassay for evaluation of potential germination stimulants for seeds of parasitic weeds. J. Pl. Growth Regul. 11:91-98.

Parker, C. 2009. Observations on the current status of *Orobanche* and *Striga* problems worldwide. Pest Manag Sci 65:453–459.

Spring, O., and E.E. Schilling. 1989. Chemosystematic investigation of the annual species of *Helianthus*. Biochem. Sys. Ecol. 17:519-528.

Spring, O. 1991. Trichome microsampling of sesquiterpene lactones for the use of systematic studies. p. 319-345. In: N.H. Fischer, M.B. Isman, and H.A. Stafford (eds.), Modern Phytochemical Methods. Plenum Press, New York, USA.

Spring, O., D. Vargas, and N.H. Fischer. 1991. Sesquiterpene lactones and benzofurans in glandular trichomes of three *Pappobolus* species. Phytochemistry 30:1861-1867.

Spring, O., N. Heil, and B. Vogler. 1997. Sesquiterpene lactones and flavonoids in *Scalesia* species. Phytochemistry 46:1369-1373.

Thuring, J.W.J.F., G.H.L. Nefkens, and B. Zwanenburg. 1997. Synthesis and biological evaluation of the strigol analogue Carba-GR24. J. Agr. Food Chem. 45:1409-1414.

Xie, X., d. Kusumoto, Y. Takeuchi, K. Yoneyama, Y. Yamada, and K. Yoneyama. 2007. 2'-epi-orobanchol and solanacol, two unique strigolactones, germination stimulants for root parasitic weeds, produced by tobacco. J. Agric. Food Chem. 55:8067-8072.

Yabuta, G., J.S. Olsen, and T.J. Mabry. 1978. Sesquiterpene lactones from the genus Zaluzania (Compositae: Heliantheae). Rev. Latinoamer. Quim. 9:83.

Yuuya, S, H. Hagiwara, T. Suzuki, M. Ando, Y. Atsushi, K. Suda, T. Kataoka, and K. Nagai. 1999. Guaianolides as immunomodulators. Synthesis and biological activities of dehydrocostus lactone, mokkalatone, eremanthin, and their derivatives. J. Nat. Prod. 62:22-30.

Zwanenburg, B., A.S. Mwakaboko, A. Reizelman, G. Anilkumar, and D. Sethumadhavan. 2009. Structure and function of natural and synthetic signalling molecules in parasitic weed germination. Pst Mang. Sci. 65:478-491.