JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

ESTABLISHMENT OF *PHELIPANCHE RAMOSA* TISSUE CULTURE AND EFFECT OF KANAMYCIN ON CULTURE GROWTH

*Dagmara Kullačová, Radoslava Matúšová**

Address(es): RNDr. Radoslava Matúšová, PhD.,
Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, 950 07 Nitra, Slovak Republic,
E-mail: radka.matusova@savba.sk, phone number: +421 37 6943330

*Corresponding author: radka.matusova@savba.sk

doi: 10.15414/jmbfs.2015.4.special2.63-65

ARTICLE INFO

Received 27. 11. 2014
Revised 2. 12. 2014
Accepted 9. 12. 2014
Published 2. 2. 2015

Regular article

OPEN ACCESS

ABSTRACT

Orobanchaceae family includes parasitic plants that attack many important food crops. Genus *Phelipanche*, belonging to this family is considered to cause high negative impact on food production. Developing *Phelipanche* plant must establish connection with the root of host plant, from which it receives all resources needed for further development. Nowadays big effort is directed to finding a reliable strategy to control parasitic plants. *In vitro* cultures of *P. ramosa* can be genetically manipulated and used for study of genes involved in host-parasite interactions. We established *in vitro* cultures of parasitic species *Phelipanche ramosa* on solid and liquid media in parallel. The obtained results point out that development of *P. ramosa* calli was origin specific. We tested the effect of antibiotic kanamycin on *in vitro* cultures of *Phelipanche ramosa* with aim to develop system for its genetic manipulation and selection of transgenic tissue using kanamycin- resistance approach. The selection pressure of kanamycin was stronger in liquid grown cultures. However, concentrations of kanamycin tested (up to 250 mg.l^{-1}) did not ensured elimination of kanamycine non-resistant tissue. Tests of other candidate selection markers are currently in progress.

Keywords: *Phelipanche ramose*, parasitic plants, *in vitro*, kanamycin

INTRODUCTION

Phelipanche ramosa is by far the most widespread root parasite. Its habitat covers Central and Southern Europe, North Africa, Asia, Central America and Australia (Parker, 2009). *Phelipanche* spp. (broomrapes) belong to the obligate holoparasitic plants, because they absolutely rely on host nutrient resources. They attack agriculturally important crops like tomatoes, tobacco, rapeseed, lettuce, hemp, leguminous plants or cruciferous plants. Host exhaustion caused by weedy *Phelipanche* spp. results in huge losses of crop yield and influences final fruit quality (Aly, 2013). Many different strategies have been tested to eliminate parasite from the environment, such as manual weeding, using herbicides or host plant breeding programs for parasite resistance, but they do not provide sufficient crop protection. One of the main reason of unsuccessful fight with these parasites is their life cycle. Parasitic plants belonging to the genus *Phelipanche* have a specific life cycle consists of several independent and several host-plant dependent stages. Independent stages include germination, development of haustorium, penetration and connection to the host root. Haustorium is an invasive feeding organ, which has invaded the host and established a functional connection between host and parasite vascular system (**Bouwmeester *et al.*, 2003**). The dependent part of life cycle includes formation of shoot, emergence above the soil, flowering and production of seeds. In the next growing season a new life cycle of the parasite starts again.

One of the most important aspects of the life cycle of genus *Phelipanche* is germination. After germination seeds are able to survive for a few days only unless they reach host root and establish a connection with the host, because the seeds are small, ranging 200-300 µm and they contain just a little bit resources (**Cardoso *et al.*, 2011**). Therefore the seeds developed a safety mechanism to germinate at short distance from the host root. The seeds germinate only after a (pre)conditioning period at suitable moisture conditions and temperature to become perceive to the chemical compounds exuded from the host roots, which induce their germination (**Matusova *et al.*, 2004**). These germination stimulants, called strigolactones (SLs), are derived from carotenoid pathway (**Matusova *et al.*, 2005**). Strigolactones, a new class of plant hormones (**Gomez-Roldan *et al.*, 2008**; **Umehara *et al.*, 2008**) are involved in many different biological processes. They play a role in root development (**Ruyter-Spira *et al.*, 2011**, **Koltai 2013**), in shoot architecture by inhibiting axillary bud growth (**Gomez-Roldan *et al.*, 2008**; **Umehara *et al.*, 2008**) or as a signal in the interaction of plants with arbuscular mycorrhizal fungi (**López-Ráez *et al.* 2011**). At present, many biological functions of strigolactones in plant development are discovered (for review **Brewer *et al.*, 2012**). Due to the discovered endogenous functions of SLs in plants, the strategy to eliminate biosynthesis of these germination stimulants had to be revised.

Nowadays, more effort is oriented to study parasitic plant-host plant interactions in laboratory conditions. The transcriptome sequencing of selected parasitic plants (Parasitic Plant Genome Project, http://ppgp.huck.psu.edu) provides excellent basis for genomic approach in study of parasitic plant-host plant interactions. However, the effective protocols for genetic transformation and regeneration of transgenic plants belonging to holoparasitic *Orobanchaceae* are still missing, partly due to specific requirements of parasitic plants for their nutrition. Studies based on *in vitro* growth and regeneration of *Phelipanche* species are considered as challenging because holoparasitic plants require a host plant for normal development (**Zhou et al., 2004**).

The success and efficiency of genetic transformation depends on the tissue culture system and methods used. In 2011, **Fernández-Aparicio *et al.*** published first protocol for genetic transformation of closely related holoparasitic species *P. aegyptiaca*. To our knowledge, genetic transformation and regeneration protocol for parasitic *P. ramosa* was not published yet. The present paper describes the establishment of *in vitro* calli cultures of parasitic plant *P. ramosa* on solid and liquid media and evaluation of the effect of antibiotic kanamycin on *in vitro* growth of *P. ramosa*.

MATERIAL AND METHODS

Seeds of *Phelipanche ramosa* (L.) Pomel were collected from vicinity of host plants tomatoes, rapeseed and tobacco and stored in lab conditions in darkness before use. Surface sterilization of *P. ramosa* seeds in 2 % (v/v) solution of sodium hypochlorite containing 0,02 % Tween-20 for 5 min with constant agitation and rinsing several times with demineralised water was sufficient to prevent contamination. *Phelipanche* seeds, approximately 120 each, were sown on 8 mm (diameter) glass fibre filter paper discs. Six such discs were then placed in 9 cm Petri dishes lined with two layers of filter paper wetted by 2.4 ml of demineralised water. The Petri dishes were sealed with Parafilm and covered by aluminium foil to provide absolute darkness. Petri dishes were then placed to the growth chamber at 22°C for seed conditioning. After 12 days of conditioning phase, the discs were transferred to new Petri dishes. To induce seed germination, on each disc 40 µl of the 0.001 mg.L^{-1} synthetic analogue GR24 was applied.

Ring of wet filter paper in Petri dish prevented drying seeds on the discs during germination. Petri dishes were wrapped with aluminium foil and seeds were incubated at 25°C in darkness for 3 days. All used items such as demineralised water and filter paper were sterilized by autoclaving, except filter-sterilised GR24.

After 3 days of GR24 exposure, germinated seeds were carefully transferred to Petri dishes containing B5 medium including vitamins (**Gamborg *et al.*, 1968**) on a filter paper support. The medium was enriched with 600 mg.L^{-1} casein hydrolysate, 5 % coconut water, 3 % sucrose, 1 mg.L^{-1} IAA and 8 mg.L^{-1} GA_3 basically according to the **Zhou *et al.* (2004).** 3.6 % potato dextrose agar was replaced by 0.8 % plant agar. Four weeks old calli of *P. ramosa* were used to test resistance to antibiotic kanamycin. Calli were transferred to liquid (without plant agar) and solid B5 medium (described above) supplemented with 0, 100, 150, 200 and 250 mg.L^{-1} of kanamycin. Each concentration of antibiotic was tested in four repetitions and each repetition contained 25 calli. Flasks with liquid cultures were placed on shaker with constant agitation at 90 rpm. All cultures were maintained at 25˚C in darkness. Calli were subcultured on kanamycin supplemented media in regular intervals.

RESULTS AND DISCUSSION

The aseptic *in vitro* culture of *Phelipanche ramosa* and infection of roots of host plants in *in vitro* conditions are useful tools to study host-parasite interaction. We studied development of *P. ramosa* under *in vitro* conditions. First, synthetic analogue of strigolactones GR24 was used for *P. ramosa* germination. The natural strigolactones are synthetized by plants in very low amounts and their isolation is extremely difficult. Therefore in laboratory experiments synthetic analogue of strigolactones GR24 is frequently used to induce germination of seeds. Based on preliminary experiments, germination of conditioned seeds was induced by 0.001 mg.L^{-1} of GR24. Application of GR24 resulted in 50-80 % germination, depending on seed origin. Germinated seeds with developing roots were transferred to plant tissue B5 medium. **Zhou *et al.* (2004)** indicated that *P. ramosa* calli were hard, white to brown with many root-like protrusions and haustorium-like structures. We noticed that further development of germinated seeds to the healthy-looking calli culture is origin dependant. After 25-30 days the germinated *Phelipanche* seeds developed tubercle-like swelling to a diameter ≥ 3.0 mm and seedlings rapidly developed into hard white calli. Seeds collected from the rapeseed host (with higher germination rate, 80 %) developed short radicles and subsequently calli of characteristic small size with necrotic parts. Seeds collected from the tomato host (with lower germination rate, about 50 %) developed several longer radicles and further developed into beige fast growing calli on solid media. Calli developed from *Phelipanche* seeds collected from tobacco field turned necrotic, with no further development.

Establishment of plant tissue *in vitro* cultures and subsequent regeneration of tissue into whole plant are one of the prerequisites for successful genetic transformation of plants (**Yildiz, 2012**). Most of genetic transformation vectors contain genes for resistance to antibiotics, which are used to select transgenic tissue. Among them, kanamycin is the selective agent the most frequently used (**Chi-Manzanero *et al.*, 2010**). The aminoglycoside kanamycin, acting as a selective agent, has been commonly used in plant genetic engineering. The *npt* II gene encodes neomycin phosphotransferase II, which allows resistance on its host cells to a wide range of aminoglycosides antibiotics such as kanamycin, neomycin, geneticin and paromomycin. Transfer of this gene together with gene of interest allows selection of transformed plant cells by growing on medium containing appropriate antibiotics. For further experiments, we used *P. ramosa* calli collected from the tomato host, developing white fragile calli with several adventitious root-like structures. In preliminary experiments we found out that growth and development of many *P. ramosa* calli were not inhibited by 150 mg.L^{-1} kanamycin concentrations. However, the growth of some calli was reduced and some of the calli necrotised (46 %) compared to the control and despite dwarfed growth some of the calli were still able to survive. Similar results achieved **Fernández-Aparicio *et al.* (2011)** for *Phelipanche aegyptiaca* using kanamycin at concentrations 50 and 100 mg.L^{-1}. In subsequent experiment, four weeks old calli of *P. ramosa* were placed to the fresh liquid and solid B5 medium (modified from **Zhou *et al.*, 2004**) supplemented by kanamycin at concentrations 0, 100, 150, 200 and 250 mg.L^{-1}. Calli were subcultured every week and on the fourth week the overall influence of antibiotic to the growth of calli was evaluated. We observed differences in morphology of calli depending on seed origin and media composition. Calli growth on solid medium was gradually reduced during the cultivation. With increasing level of added antibiotic the growth of calli was remarkably inhibited. At the concentration of kanamycin 250 mg.L^{-1}, calli were obviously smaller, darker, brown, most of them were necrotic (69 %) compared to the control. After 28 days of cultivation control samples looked fresh with big biomass of calli, which were white or beige (Fig. 1). Calli culture in liquid medium behaved in similar way, and at the highest kanamycin concentration all the calli were necrotic in every repetition (Fig.1).

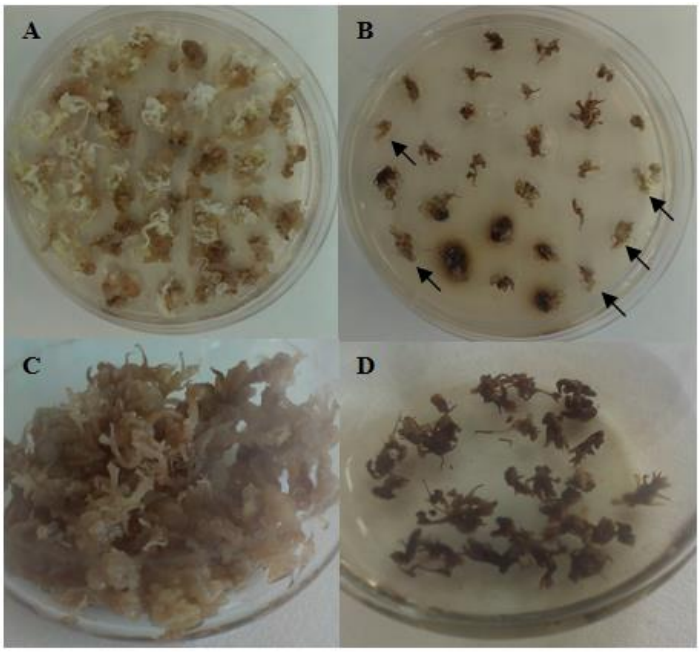

Figure 1 The effect of kanamycin on *Phelipanche ramosa* calli. (A) control calli on solid medium after 28 days of cultivation, (B) calli on solid medium supplemented with 250 mg.L^{-1} of kanamycin after 28 days (arrows show healthy looking calli despite their limited increase of biomass), (C) control calli from liquid medium after 28 days, (D) calli in liquid medium supplemented with 250 mg.L^{-1} of kanamycin after 28 days all explants necrotised.

Kanamycin at range of tested concentrations 0-250 mg.L^{-1} on solid medium did not cause death of all calli, some of them were able to grow under this relatively high kanamycin concentration. In one case, all calli grown on medium supplemented with 250 mg.L^{-1} of kanamycin turned necrotic, brown and the process was accompanied by lysis of cells. In liquid medium supplemented with 250 mg.L^{-1} of kanamycin, all the calli were necrotic after 28 days of cultivation. It is obvious, that the selective pressure of antibiotic was stronger in liquid environment than on solid medium. This can be explained by whole surface exposition of calli to selective agents. The aminoglycoside kanamycin is commonly used to select plant cells transformed with the neomycin phosphotransferase marker gene. This antibiotic has inhibitory effect on cell growth at lower concentrations (50-100 mg.L^{-1}) in many plant species, but some plants are able to tolerate higher concentration of antibiotics in the environment. In several cases, unusually high concentrations of antibiotics were used. **Parveez *et al.* (2007)** reported high endogenous resistance of oil palm immature embryos to the 2000 mg.L^{-1} of kanamycin in media, which resulted in inhibition of their growth by 15 % only.

CONCLUSION

We established *in vitro* cultures of parasitic plant *Phelipanche ramosa* on solid and liquid media and evaluated the effect of antibiotic kanamycin on growth and morphogenesis of parasite tissue. The growth of *P. ramosa* calli upon high concentration of kanamycin suggests that selection of *P. ramosa* tissue on the basis of resistance to kanamycin might be not effective enough to eliminate non-transgenic cells. Alternative approach to antibiotic resistance marker genes might be the use of non-destructive markers to identify transgenic tissue of *P. ramosa*. Non-destructive fluorescent markers e.g. green (GFP), yellow (YFP) or red (DsRED) fluorescent proteins are good candidates to be more efficient selection markers for *Phelipanche ramosa*.

Established *in vitro* cultures of *Phelipanche* spp. might also be used in screening of herbicides. In case of holoparasitic *Phelipanche* and *Orobanche* spp., herbicides must be applied during underground development **(Eizenberg *et al.* 2013)** and *in vitro* cultures of *Phelipanche* represent such developmental stages.

Acknowledgments: This research was supported by the Slovak Grant Agency VEGA (project 2/0139/11), Slovak Academy of Sciences for *COST Action FA1206* Strigolactones: biological roles and applications, *COST Action FA1206* and by European Community under project no 26220220180: Building Research Centre „AgroBioTech".

REFERENCES

ALY, R. 2013. Trafficking of molecules between parasitic plants and their hosts. *Weed Research*, 53, 231-241. http://dx.doi.org/10.1111/wre.12025

BOUWMEESTER, H. J., MATUSOVA, R., ZHONGKUI, S., BEALE, M. H. 2003. Secondary metabolite signalling in host-parasitic plant interactions. *Current Opinion in Plant Biology*, 6(4), 358-364. http://dx.doi.org/10.1016/s1369-5266(03)00065-7

BREWER, P.B., KOLTAI, H., BEVERIDGE, C.A. 2012. Diverse roles of strigolactones in plant development. *Molecular Plant*, 6(1), 18–28. http://dx.doi.org/10.1093/mp/sss130

CARDOSO, C., RUYTER-SPIRA, C., BOUWMEESTER, H. J. 2011. Strigolactones and root infestation by plant-parasitic *Striga*, *Orobanche* and *Phelipanche* spp. *Plant Science*, 180(3), 414-420. http://dx.doi.org/10.1016/j.plantsci.2010.11.007

EIZENBERG, H., HERSHENHORN, J., EPHRATH, J.H., KANAMPIU, F. 2013. Chemical control, 415-432. In: JOEL, D.M., GRESSEL, J., MUSSELMAN, L. J. (eds.) Parasitic Orobanchaceae, Springer-Verlag Berlin Heidelberg 2013. http://dx.doi.org/10.1007/978-3-642-38146-1_23

FERNÁNDEZ-APARICIO, M., RUBIALES, D., BANDARANAYAKE, P., YODER, J., WESTWOOD, J. 2011. Transformation and regeneration of the holoparasitic plant *Phelipanche aegyptiaca*. *Plant methods*, 7(36), 1-10. http://dx.doi.org/10.1186/1746-4811-7-36

GAMBORG, O. L., MILLER, R. A., OJIMA, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50(1), 151-158. http://dx.doi.org/10.1016/0014-4827(68)90403-5

GOMEZ-ROLDAN, V., FERMAS, S., BREWER, P.B., PUECH-PAGES, V., DUN, E. A., PILLOT, J.-P., LETISSE, F., MATUSOVA, R., DANOUN, S., PORTAIS, J.-C., BOUWMEESTER, H., GUILLAUME, B., BEVERIDGE, CH. A., RAMEAU, C., ROCHANGE, S. F. 2008. Strigolactone inhibition of shoot branching. *Nature*, 455, 189–194. http://dx.doi.org/10.1038/nature07271

CHI-MANZANERO, B. H., ACERETO-ESCOFFIÉ, P. O. M., CASTAÑO, E., RODRÍGUEZ-ZAPATA, L. C. 2010. Optimal concentration of kanamycin as a selective agent for the transformation of *Musa* cv. "GRAND NAIN". *Universidad y Ciencia*, 26(1), 115-119.

KOLTAI, H. 2013. Strigolactones activate different hormonal pathways for regulation of root development in response to phosphate growth conditions. *Annals of Botany*, 112(2), 409-415. http://dx.doi.org/10.1093/aob/mcs216

LÓPEZ-RÁEZ, J.-A., CHARNIKHOVA, T., FERNÁNDEZ, I, BOUWMEESTER, H, POZO, M. J. 2011. Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato. *Journal of Plant Physiology*, 168(3), 294–297. http://dx.doi.org/10.1016/j.jplph.2010.08.011

MATUSOVA, R., RANI, K., VERSTAPPEN, F. W. A., FRANSSEN, M. C. R., BEALE, M. H., BOUWMEESTER, H. J. 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. *Plant Physiology*, 139(2), 920-934. http://dx.doi.org/10.1104/pp.105.061382

MATUSOVA, R., VAN MOURIK, T., BOUWMEESTER, H. J. 2004. Changes in the sensitivity of parasitic weed seeds to germination stimulants. *Seed Science Research*, 14(4), 335-344. http://dx.doi.org/10.1079/ssr2004187

PALLAVI, M., GOSAL, S. S., SENGER, A., KUMAR, P. 2009. Impact of cefotaxime on somatic embryogenesis and shoot regeneration in sugarcane. *Physiology and Molecular Biology of Plants*, 15(3), 257-265. http://dx.doi.org/10.1007/s12298-009-0029-3

PARKER, C. 2009. Observations on the current status of *Orobanche* and *Striga* problems worldwide. *Pest Management Science*, 65(5), 453-459. http://dx.doi.org/10.1002/ps.1713

PARVEEZ, G. K. A., MAJID, N. A., ZAINAL, A., RASID, A. 2007. Determination of minimal inhibitory concentration of selection agents for selecting transformed immature embryos of oil palm. *Asia Pacific Journal of Molecular Biology and Biotechnology*, 15(3), 133-146.

RUYTER-SPIRA, C., KOHLEN, W., CHARNIKHOVA, T., VAN ZEIJL, A., VAN BEZOUWEN, L., DE RUIJTER, N., CARDOSO, C., LOPEZ-RAEZ, J.-A., MATUSOVA, R., BOURS, R., VERSTAPPEN, F., BOUWMEESTER, H. 2011. Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: Another below-ground role for strigolactones? *Plant Physiology*, 155(2), 721-734. http://dx.doi.org/10.1104/pp.110.166645

UMEHARA, M., HANADA, A., YOSHIDA, S., AKIYAMA, K., ARITE, T., TAKEDA-KAMIYA, N., MAGOME, H., KAMIYA, Y., SHIRASU, K., YONEYAMA, K., KYOZUKA, J., YAMAGUCHI, S. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature*, 455(7210), 195–200. http://dx.doi.org/10.1038/nature07272

YILDIZ, M.: The prerequisite of the success in plant tissue culture: High frequency shoot regeneration. In: LEVA, A. – RINALDI, L.M.R.: Recent advances in plant *in vitro* culture. 2012. InTech, 220 p., ISBN 978-953-51-0787-3. http://dx.doi.org/10.5772/51097

ZHOU, W. J., YONEYAMA, K., TAKEUCHI, Y., ISO, S., RUNGMEKARAT, S., CHAE, S. H., SATO, D., JOEL, D. M. 2004. *In vitro* infection of host roots by differentiated calli of the parasitic plant *Orobanche*. *Journal of Experimental Botany*, 55(398), 899-907. http://dx.doi.org/10.1093/jxb/erh098