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

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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 parasitic 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 of 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 (Ruiter-Spira et al., 2011, Kolta 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\(^{-1}\) G6P. Seeds were germinated according to the Zhou et al. (2004), 3.6 % potato dextrose agar was replaced by 0.8 % plant agar. Four weeks old calli of \textit{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 \textit{Phelipanche ramosa} and infection of roots of host plants in \textit{in vitro} conditions are useful tools to study host-parasite interaction. We studied development of \textit{P. ramosa} under \textit{in vitro} conditions. First, synthetic analogue of strigolactones GR24 was used for \textit{P. ramosa} germination. The natural strigolactones are synthesized 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 \textit{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 is origin dependant. After 25-30 days the germinated \textit{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, about 50 %) developed several longer radicles and subsequently calli of characteristic small size with necrotic parts. Seeds collected from the tomato host (with lower germination rate, about 46 %) were placed to the fresh liquid and solid B5 medium. All cultures were maintained at 25˚C in darkness. 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.

Establishment of plant tissue \textit{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-Manzano et al., 2010). The aminoglycoside kanamycin, acting as a selective agent, has been commonly used in plant genetic engineering. The \textit{npt II} gene encodes neomycin phosphotransferase II, which allows resistance on host cells to 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 \textit{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 \textit{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 \textit{Phelipanche aegyptiaca} using kanamycin at concentrations 50 and 100 mg.L\(^{-1}\). In subsequent experiment, four weeks old calli of \textit{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 after 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).

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. Parvez 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 \textit{in vitro} cultures of parasitic plant \textit{Phelipanche ramosa} on solid and liquid media and evaluated the effect of antibiotic kanamycin on growth and morphogenesis of parasite tissue. The growth of \textit{P. ramosa} calli upon high concentration of kanamycin suggests that selection of \textit{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 \textit{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 \textit{Phelipanche ramosa}.

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

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