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Hairy roots production in *Phyllanthus odontadenius* Müll. Arg. by seedlings transformed with *Agrobacterium rhizogenes* A4RS/pHKN29

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Phyllanthus odontadenius **Müll. Arg is one of the most important medicinal plants in the genus *Phyllanthus* (Phyllanthaceae). It is used in different regions in the world for the treatment of various diseases for example malaria caused by *Plasmodium falciparum* causing millions deaths in tropical and subtropical regions. In this work, we experimented *P. odontadenius* seeds germination and seedlings infected by *Agrobacterium rhizogenes* A4RS/pHKN9. We obtained 36% of seeds that germinated for 84.6% of seedlings transformed which showed positive *gfp* activities. The number of hairy roots formed in infectious sites for seedlings infected with *A. rhizogenes* A4RS/pHKN29 is valued at 6.3±2.71 against 0 for control seedlings. Diagrams showed that mean length for the new-formed roots were 1.92±0.55 cm and 1.59±0.49 cm for hairy root.**

Key words: *Phyllanthus odontadenius*, hairy roots, *Agrobacterium rhizogenes*, malaria, secondary metabolites.

INTRODUCTION

In many tropical and subtropical regions, malaria remains one of the main global health problems of our time, causing more than 1 million deaths per year, with about 90% of deaths and 60% cases occurring in South Africa in the Sahara. It is caused by the protozoan parasite *Plasmodium falciparum* and transmitted by female Anopheles mosquitoes, which bite mainly between sunset and sunrise (WHO, 2007; Orhan et al., 2006; Mitaine-Offer et al., 2002).

The widespread resistance of *P. falciparum* against classical antimalarial drugs through the tropics (Olliaro and Yuthavong, 1999) has led to a research of new drugs with new modes of action (Tona et al., 2004). It is well known that plant species used in traditional or folk

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medicine synthesize and accumulate various secondary metabolites (Cimanga et al., 2004). *P. odontadenius* is one of most important medicinal plants in the genus *Phyllanthus* (Phyllanthaceae) used in different regions in the world for the treatment of various diseases (Unander et al., 1991; Bajaj, 1999; Luyindula et al., 2004).

In recent years, rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious, genetically (Ri-T-DNA) transformed roots (Chabaud et al., 2006). A. rhizogenes mediated transformation has also been used to produce transgenic hairy root culture and plantlets have been regenerated (Anand, 2010). Several studies on *A. rhizogenes* (Tepfer, 1983, 1984, 1990; Chabaud et al., 2006) have shown that although many dicotyledonous plants are susceptible to infection by these bacteria and can be transformed and produce hairy roots, some of these species have no ability to regenerate plantlets through their hairy roots.

Although *Phyllanthus niruri* hairy roots have been previously reported (Ishimaru et al., 1992; Bajaj, 1999) and showed the main constituents contained in these hairy roots. The objective of this work was to compare firstly the standard methods and modifications to those methods for *P. odontadenius* seeds germination from Kinshasa in DRC (Democratic Republic of the Congo) and secondarily to investigate *P. odontadenius* hairy roots production using *A. rhizogenes* A4RS/pHKN29 for later identification or isolation of secondary metabolites against *P. falciparum* causing malaria disease.

MATERIALS AND METHODS

Plant material and culture methods

Seeds of *P. odontadenius* from CGEA/ CREN-K in Kinshasa (DRC) were subjected to six essays of germination (A, B, C, D, E and F). Firstly, seeds were surface scarified with sulfuric acid 96% and disinfected firstly with 70% (v/v) ethanol for 1 min, and then in 0.1% (p/v) aqueous mercuric chloride ($HgCl_2$) for 3 min (Zhao et al., 2006). Secondarily, seeds were dipped in 20% Domestos for 10 min followed by four rinses with sterile water. Then seeds were dipped in 200 ppm gibberellic acid (GA_3) solution during 4 h (Jimenez et al., 2007). Thirdly, H medium (Hoagland and Arnon, 1938) was prepared then adjusted to pH 5.6 and solidified with 8 g/l agar before autoclaving at 121°C for 15 min. Seeds were placed aseptically on hormone-free H medium and incubated at 27°C for 16 h photoperiod with 50 µmol/m^2/s. Leaved seedlings were used for inducing hairy roots.

Bacterial strain culture

A. rhizogenes, strain A4RS harboring pHKN29 plasmid, containing GFP only, was used as a binary vector for the generation of control hairy roots (Kumagai and Kouchi, 2003; Nakatsukasa-Akume et al., 2005), activated by culturing on Luria and Bertani (LB) medium (LB medium: 1 L containing 10 g Bacto-tryptone, 5 g of yeast extract, 10 g of sodium chloride salt and 15 g of agar with pH adjusted to 7.2 before autoclaving). 200 mg/l of kanamycin and 300 mg/l of streptomycin were prepared for incorporation. Previously, bacteria suspensions were prepared using single colonies of bacteria in LB medium supplemented with related antibiotics and cultured overnight at 28°C shaken at 120 rpm (Zhao et al., 2006) .The cultured A4RS/ pHKN29 was transferred on LB agar for 1 or 2 days infection later on hypocotyl *Phyllanthus* seedlings.

Maintenance of hairy root cultures

Axenic *P. odontadenius* seedlings were infected directly with *A. rhizogenes* strain A4RS/ pHKN29 using a needle G26. Each of the infected seedlings was cultured on solid MS/2 medium (Murashige and Skoog, 1962; Saitou et al., 1999; Bhattacharyya and Bhattacharya, 2004) and incubated in darkness. After the first appearance of hairy roots, seedlings were transferred to hormone-free solid MS/2 medium gelled with 0.8% agar containing 3% sucrose and 500mg/l cefotaxime. About three weeks after infection, several hairy roots appeared at the wounded sites. These were cut off and cultured in hormone-free MS/2 semi-solid medium containing 500 mg/l cefotaxime for one week. This process was repeated three times to eliminate completely bacteria from the transformed roots (Ridgway et al., 2004). For investigation, number of hairy roots was counted seedling by seedling. They were cut and transferred on M/2 medium in square plate for growth.

Localization of hairy root gfp activity and calli produced at hypocotyl plants

Seedlings observation was made directly on seedling cultivated in Petri dish (plates) on solid MS/2 medium incubating after 2 weeks with bi-ocular lens MZFIII (Leica). The filters used were the *GFP1* (excitation 480/40 nm; stopped: 510 nm).

Number and length measurement

Number and length for transformed roots or untransformed roots (10) were measured with OPTIMAS 6.1 programs. Means were determined for each measurement.

RESULTS AND DISCUSSION

Seeds germination of *P. odontadenius*

Table 1 shows 36 ± 16.59% as total number of *P. odontadenius* seeds that germinated and seeds not dipped in GA_3 that did not germinate. 36±16.59% divided as well as 76.4% for Jimenez *et al.,* 2007 protocol (F) and 23.6% for *Casuarina* Protocols (E). 41.87% of seedlings from Jimenez et al. (2007) protocols were infected with *A. rhizogenes* A4RSpHKN29 with 5.4% only for seedlings from *Casuarina* protocols. On the other hand, 4.13% of seedlings obtained were immature for their infection with *A. rhizogenes* A4RSpHKN29. Others protocols used have not produce germination or seedlings because sulphuric acid showed a fatal effect on *P. odontadenius* seeds germination and on the other hand, gibberellic acid (GA_3) has positive or stimulate effect in raising of

Table 1. Percentage of *P. odontadenius* seeds germination.

Protocol types	Percentage of germination (%)	Percentage of seedlings infected with A4RSpHKN29 (%)	Percentage of seedlings not infected with A4RSpHKN29 (%)	Percentage of control seedlings (%)	Percentage of immature seedlings (%)
F	76.4	41.87	16.50	13.91	4.13%
E	23.6	5.4	7.3	0	10.9%
Total	36±16.59% of seeds germinated in the total				

F: Protocol F: Protocol of Jimenez et al. (2007); E: Protocol E: Protocol of *Casuarina (*Rhizogenesis Laboratory, IRD)*; Gfp*: Green fluorescent proteins; A4RSpHKN29: *Agrobacterium rizogenes s*train A4RS harboring pHKN29 plasmid.

Table 2. Distinctive morphological features for seedlings infected with A4RSpHKN29.

Parameter	Wounded seedlings	Presence of calli	Absence of calli	Seedlings with *gfp*	Seedlings without *gfp*
Seedlings wounded with A4RS/pHKN29	100%	73.10%	26.90%	84.60%	15.40%
Seedlings not wounded with A4RS/pHKN29	100%	0	100%	0	100%

GFP: Green fluorescent protein.

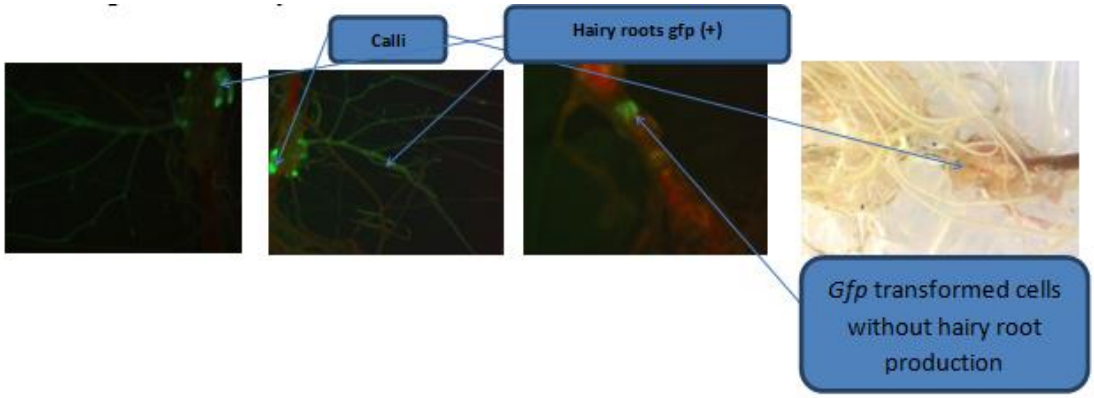

Figure 1. Enzymatic evidence and callus transformation.

dormancy seeds (Tourte, 2002).

Localization of hairy root gfp activity and calli produced on hypocotyl plantlets

Observations made under LEICA microscopic in order to certify gene reporter expression of gfp are illustrated in Table 2 and Figure 1.

Table 2 shows that 73.1% plantlets wounded with *A. rhizogenes* A4RSpHKN29 have produced calli on infected sites against 26.9 and 84.6% seedlings presenting positive gfp on the infected sites against 15.4% with negative *gfp* on the infected sites with *A. rizogenes*A4RS/pHKN29. All seedlings wounded without *A. rizogenes*A4RS/pHKN29 did not present calli or *gfp* on the infected sites. Figure 1 show enzymatic evidence and callus transformation.

Hairy roots counting and length measurement

Hairy roots number by seedlings in the infected sites and their length are presented in Figures 2 and 3. Figure 2a shows that the infected plantlets produced most new-formed roots with 6.17±3.37 in the wounded sites of *P. odontadenius* seedlings against 4.83±1.83. These two values did not present difference at P=5% (F-cal. = 0.73

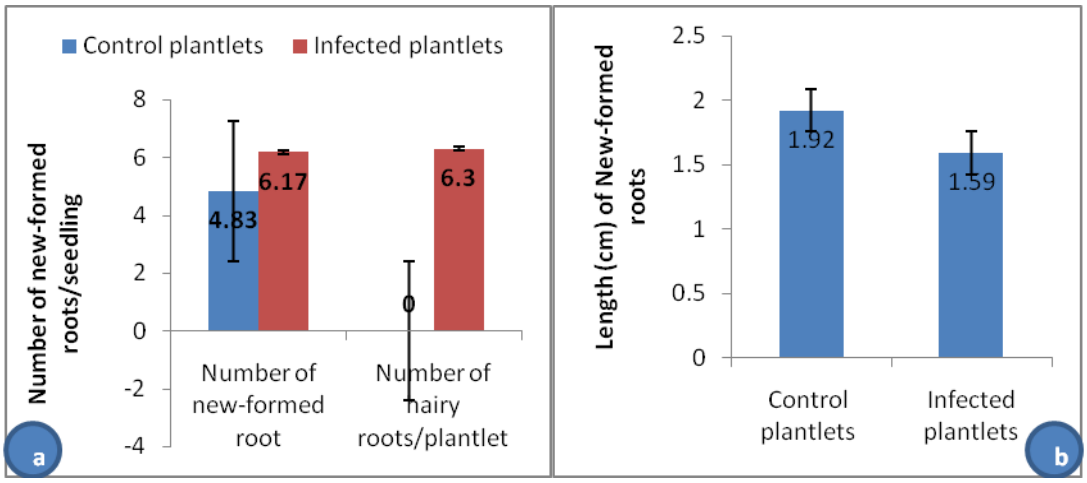

Figure 2. Number of newly-formed roots on plantlets wounded sites and length of newly-formed root on the wounded sites of *P. odontadenius.*

Figure 3. Autonomous growth of hairy roots on MS/2 in square disk.

< F-Tab. = 5.05). Hairy roots obtained with infected plantlets by *A. rhizogenes* A4RSpHKN29 were 6.3±2.71 against zero for control seedlings.

Figure 2b shows that control seedlings produced newly-formed roots in the wounded sites which measured 1.92±0.55 cm against 1.59±0.49 cm. These two values do not differ at P=5% (F-cal. = 0.97 < F-Tab. = 5.05).

Proof of autonomous hairy roots growth

Hairy roots obtained by infection of *P. odontadenius* seedlings were cut and transferred into the square disk for their autonomous growth in on MS medium with cefotaxime 300 mg/ml. The growth of hairy roots is illustrated in the Figure 3.

Figure 3 shows that all hairy roots (1, 2, 3 and 4) marked respectively (a-a'), (b-b'), (c-c') and (d-d') placed on MS/2 in square plate were increasing in length. The means length was 0.88±0.62 cm as presented in Figure 4.

DISCUSSION

The main objective of this investigation was to establish protocol of germination and hairy roots production for *P. odontadenius*, an important herbal medicinal plant. In this work, we have been showed on one hand, the role of gibberellic acid in seeds germination or seeds lifting dormancy and embryo lifting dormancy (Tourte, 2002). On the other hand, the sulphuric acid toxicity when it is used in *P. odontadenius* seeds scarification which did not germinate after their dipping in gibberellic acid solution 200 ppm. Contrary to the results obtained by Jimenez et al. (2007) with the higher percentage (60%) of *P. niruri* seeds germination *in vitro*, we obtained 36% of germinated seeds. These results are better than the various results (3.8 - 30.20%) obtained by Unander et al. (1995) after the experiments on factors affecting germination. Authors reported that the hours after harvest, the temperature, hours of exposure to light and the environment of seeds derived are the important factors in *P. amarus* seeds germination.

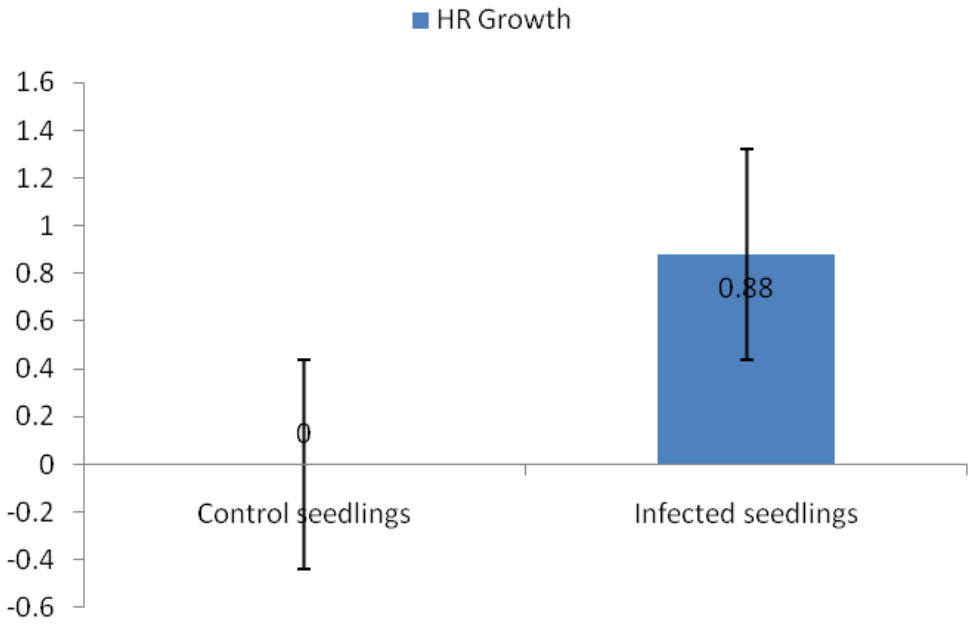

Figure 4. Length of increasing hairy roots on MS/2 in square disk.

The variation in our results shows the explanation in Unander et al. (1995) and by the Kinshasa environment issue (DRC) and the cultured media (MS and H). Another fact, we have obtained new-formed roots with all seedlings, wounded with needle and infected or uninfected with *A. rhizogenes*. It can be caused by the stress due to wounds by needle and by gibberellic acid used in seeds germination. These factors will be active on auxin hormone tenor which is increasing frequently whether by synthesis stimulations or by auxin - oxydase inhibitions for the uninfected seedlings (Augé et al., 1984). Then basic peroxydases have been particularly involved in the metabolism of auxinique catabolism, the application of gibberelic acid has been known in the increase of endogenous auxins (Zrÿd, 1988).

A. rhizogenes A4 strain, resistant to rifampicine and spectinomycine, have been shown their efficiency in the hairy roots production in Coffee (*Coffea arabica*) (Alpizar et al., 2008). Pirian et al. (2012) reported that the A4 strain was less efficient than AR15834, 9534 and C318 in the hairy root induction of *Portulaca oleracea* for adrenalin production. All strains used by these authors were rifampicin resistant.

Further, the infected or uninfected seedlings have been distinguished by the presence of callus, principle characteristic of *A. rhizogenes* strain A4RS/ pHKN29 and the green fluorescent protein (*gfp*) activities produced by the reporter gene coding *gfp* (Kumagai and Kouchi, 2003; Nakatsukasa-Akume et al., 2005) in the seedlings infected by A4RS/pHKN29. Another characteristic was the growth of hairy roots on MS/2 in the square plate than the new-formed root of control seedlings.

However, this experiment did not show difference between number and length of hairy roots (Figure 2) obtained and new-formed roots from seedlings of *P. odontadenius* uninfected with A4RS/pHKN29. This experiment has also noticed the difference in the length of the roots produced by seedlings of *P. odontadenius* and the non-multiplication of roots from the non-tainted seedlings when they were put in cultures on MS/2 without phyto-hormones whereas this growth is manifested for the roots from tainted seedlings with A4RS/pHKN29.

Conclusion

This survey revealed that *A. rhizogenes* A4RSpHKN29 encouraged the transgenic root production when the seedlings of *P. odontadenius* from germination of seeds (36%) were soaked beforehand in the gibberellic acid (200 ppm) for four hours. The proof of effectiveness of transformation has first been shown by the presence of the calluses instead of infection and then by the positive *gfp* activity when those roots are observed under the luminescent microscope. The growth of hairy roots on MS/2 in square disk was also the characteristic which confirm transgenic root production in *P. odontadenius.*

Conflict of Interest

The authors have not declared any conflict of interest.

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