In vitro host-free seed culture, callus development and organogenesis of an obligatory root-parasite *Striga hermonthica* (Del.) Benth: the witch-weed and medicinal plant

Faisal Hammad Mekky Koua,[1] Fatima Misbah Abbas,[2] Eisa Ibrahim Elgaali,[2] Mutasim Mohammed Khalafallah,[2] Hind Ahmed Ali Babiker[1]

[1]Department of Biochemistry, School of Biotechnology, Faculty of Science & Technology, Al-Neelain University, Khartoum, Sudan; [2]Commission for Biotechnology and Genetic engineering, National Center for Research, Khartoum, Sudan

Abstract

Striga hermonthica (Del.) Benth a well-known hemi-parasitic weed, it also has been used widely in African folk medicine to remedy broad spectra of diseases. The current contribution is an attempt to establish reproducible in vitro callusing system. *In vitro* seedling's stem segments were used as an explant for callus induction, in 1.5% or 3.0% sucrose added into Murashig and Skoog medium (MS) and supplemented with different auxins, α-Naphthalene-3-acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), Indole-3-acetic acid (IAA), or Indole-3-butryic acid (IBA) at different concentrations each alone or in combination with cytokinin 0.5 mgl^{-1} 6-benzyl aminopurine. The most effective auxin was NAA with maximum 79% callus induction rate. All auxin treatments induced callus in all concentration when used alone or combined with BAP, except 2,4-D, which induced the callus only when combined with BAP. A high sucrose concentration was required for high callus induction rate by all auxin treatments. IAA and IBA auxins induced direct shoot regeneration and had low callus induction rates. NAA, IAA and IBA induced organogenic calli, whereas 2,4-D in combination with BAP induced non-organogenic callus. We further screened preliminarily the phytochemical contents of the callus and intact plant, which was revealed the presence of flavonoids, terpenes, saponins, cardiac glycosides, alkaloids, tannins and coumarins. Experimental data of both seed culture and callus induction could provide a route to further enhance the efficiency of callus initiation of *S. hermonthica* for medicinal purposes and understanding the infection mechanism of the witch-weed plant.

Introduction

Striga hermonthica (Del.) Benth, an obligatory root-parasitic plant, which causes considerable yield loses amongst many graminaceous cereal crops of great importance as food crops, e.g., rice (*Oryza sativa* L.), millet *(Pennisetum glaucum* L. Leeke), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) roots. It belongs to the family Scrophulariaceae,[1-4] and it's widespread in West and East Africa.[5-7] On some parts of Africa S. hermonthica is used as medicinal plant for treatment of leprosy and leprous ulcers.8 In East Africa, a decoction or infusion of the roots is administered orally as an abortifacient and in the treatment of pneumonia as well.[9] According to previous report *S. hermonthica* extract has revealed an antimalarial activity.[10] It has also been revealed that this plant has *in vitro* trypanocidal effect against *Trypanosoma congolense* and *Trypanosoma cruzi*.[11] It's known that natural habitats for medicinal plants are disappearing very fast due to environmental and geopolitical instabilities; therefore, difficulty to acquire plant-derived compounds is increasing.[12] An alternative to field-grown crops or to chemical synthesis has been large-scale *in vitro* plant tissue culture.[13] *In vitro* cultures may potentially constitute useful and easily manipulated systems for producing valuable biologically active compound in plants that do not require labor-intensive methods. The study of biochemical and physiological features of parasites and host-parasite interaction requires the development of an *in vitro* infection system, free of microorganism contamination, with full regeneration of the parasite. Plant tissue culture offers several advantages over traditional whole plant systems to study the molecular and biochemical aspects of host-pathogen interactions.[14] Nevertheless, growing these parasitic plants *in vitro* is difficult, because of their dependence on connection to hosts for normal development and because of their specific germination requirements.[14-16] There has been only one report of *S. hermonthica* calli using the fresh leaves as explants,[17] here, for the first time we successfully induced the *S. hermonthica* callus induction and shoot regeneration directly or from the produced calli using *in vitro* stem segments as explants for both callogenesis and shooting. The current study was undertaken with a primary objective of establishing and defining an effective *in vitro* system for callus induction using MS media treated with several natural and synthetic auxins each alone or in combinations with BAP. While metabolic profiling in plants is increasing, such studies have not been carried out yet on a parasitic plant, and information on the biochemistry of *S. hermonthica* remains poor.[17] Our current established callus induction system is suitable for the comparative metabolomics studies toward understanding the infection mechanisms by inducing an *in vitro* infection and for the folkloric medicinal applications as well.

Correspondence: Faisal Hammad Mekky Koua, Department of Biochemistry, School of Biotechnology, Faculty of Science & Technology, Al-Neelain University, P O Box 12702, Al-Baladya St., Khartoum, Sudan.
Tel. +249.183.780058.
E-mail:faisalkoua@gmail.com

Key words: callus induction, germination stimulant, parasitic plant, seed germination, *Striga* hermonthica.

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Materials and Methods

Plant materials

S. hermonthica seeds collected from the Sorghum Crop fields of Gazira, Sudan (Season 2003), were kindly supplied by Prof. Babiker AGT, Agricultural Research Corporation, Gezira State, Sudan. The seeds were sterilized using commercial Clorox 5% (v/v) (active ingredient sodium hypochlorite) and prepared for tissue culture experiments as described previously.[18] The intact plants were collected as whole plants from Shambat forage field, Khartoum, Sudan.

GR24 stimulant and MS media preparation

GR24 was kindly provided by Prof. Babiker AGT. 1 mg of GR24 powder was dissolved in 1 mL acetone, and then made up to 100 mL by distilled water and filter sterilized. Murashige and Skoog (MS) basal medium was prepared with 1.5% and 3% sucrose at pH 5.8 and used for the planting of *S. hermonthica* germinated seeds.[19]

Seed pre-conditioning and germination

The sterilized seeds were soaked in 20 mL of sterile distilled water in sterile Petri dishes lined with sterile filter paper and incubated in a dark condition at 25±2ºC for 16 days. During this period the water was changed daily and in day 16 the seeds were transferred and placed onto new sterile Petri dishes lined with sterile filter paper then soaked with filter sterilized 10 μg L^{-1} GR24, incubated in dark conditions at 25±2ºC for other 48-72 h, and then the seeds were examined for radicle emergence under a dissecting microscope (3X).[18]

Seed transfer on MS medium and *in vitro* seedling production

In order to produce *in vitro* seedlings for subsequent experiments, germinated seeds were transferred into previously prepared MS culture media using sterile needles under entirely sterile condition and then incubated in a cultivation chamber under fluorescent-light of 16h/day at 25±2.0ºC.[18] This step was performed under a dissecting microscope (3X) in order to distinguish and pick the germinated seeds.

Callus induction and organogenesis

The stems of 4-weeks age seedlings grown on the MS basal medium were excised with sterile blade under highly sterile conditions to a length of 1.0 cm and used as explants.[18] MS basal medium was supplemented with various auxins, NAA, 2,4-D, IAA, IBA at 1.0, 1.5, 2.0 and 4.0 mg L^{-1} concentrations used alone or in combination with BAP 0.5 mg L^{-1} for callus induction and subsequent shoots and root-like structures generation. The cultures were incubated under fluorescent light for 16 h/day at 25±2ºC. Each experiment had 10 replicates and was repeated at least three times to a minimum sample size of 30 replicates. Callus induction was scored 4-weeks after culturing. An explant with unorganized cell clusters growing at least 1 mm in size was considered *callogenesis*. For shoot regeneration, stems segments and organogenic calli of IAA, IBA and NAA at different concentrations were used to generate shoots and root like structures. Transferring the callus or the stem segments into a MS medium containing NAA, IAA or IBA or instead incubating the callus more than 4-weeks for NAA or 2-weeks for IAA and IBA generate shoots and root-like structures. The organogenesis were used for the maintenance of the plant *in vitro* and the mature plant of 4-week were sub-cultured continuously during the period of this study.

Phytochemical screening

The intact plant samples were divided into aerial (shoots) and underground (haustoria) parts and air-dried at room temperature, then blended and stored till used. The NAA's calli of 1.0 mg L^{-1} and 3.0% sucrose were harvested at 4-weeks age and freeze-dried and stored as powder till used. A successive soxhlet extraction of petroleum ether (35-40ºC) and ethanol followed by a water extraction by soaking the ethanol (99.8%) extract's marc in sterile distilled water for 24.0 h were applied. The phytochemical constituents of the intact plant and calli extracts were appraised, of which the presence of triterpenes, sterols, tannins, coumarins, cardiac gylcosides, flavonoids, saponins, anthrocenosides, carotenoids, glycosides and alkaloids were detected following the standard protocols.[20-22]

Statistical analysis

The data were analyzed with SPSS software (version 11.5). A one-way analysis of variance (ANOVA) was implemented to test the effects of 1.5 and 3.0% concentration of sucrose and the influence of growth regulator supplements on callus induction. The significance was determined at a 5% level by using the Duncan's multiple range tests.

Results and Discussion

The surface sterilized seeds were germinated aseptically for the production of *in vitro* seedlings, which were used later as explants in callus induction experiments and for shoot maintenance. The complex sorghum root exudates were replaced with a synthetic germination stimulant GR24,[23] and the majority of the seeds were stimulated for germination within 16 days by producing transparent radicles, in consistent with.[24] Since the induction of *S. hermonthica* callus is the main target of this study, a head of this study the seeds and seedling's stem were tested for their ability to induce callus in MS medium. MS medium as reported previously was showed suitability for *in vitro* growth response of this parasitic plant.[25] The *in vitro* fresh seedling stem was selected as most effective explant for callus induction for this study (*data not shown*). In vitro multiple shoots were maintained by subculturing the regenerated stem segments every four weeks in MS basal medium without adding growth regulators in order to maintain the seedlings. A previous report states that the growth of parasitic seedlings does not need an addition of growth requirements (MS-vitamins, casein hydrolysate and meso-inositol) that are needed by non-parasitic plants.[26] It also has been reported that *S. asiatica* seedlings can develop on simple, inorganic, defined media with salts and sucrose only, and without the aforementioned growth requirements.[27,28] However, in the same studies it has been noticed that in some cases the growth was better when vitamins, casein-hydolysate and myo-inositol were included.[14,25,27] Nevertheless, the above growth requirements were used in the callogenesis study of S. hermonthica.[17] In this study, due to the debates about the growth requirements regarding the *in vitro* culture development of *S. hermonthica*, we based on the general MS medium constituents for plant *in vitro* culture.[17,19]

Influence of auxins growth regulators and sucrose concentration on callus induction

Auxins alone and in combination with cytokinins have been extensively studied to enhance callus induction and maintenance.[29] Experiments to investigate the effects of both plant growth regulators and sucrose concentration on the induction rate of *S. hermonthica* callus were carried out. In these experiments, four different auxins NAA, 2,4-D, IAA and IBA were used seeking for induction of healthy calli with high induction rate. Different concentrations of auxins were implemented and two different sucrose concentrations were used as well. The highest callus induction rate was obtained when the MS medium treated with NAA at concentration of 1.0 mg L^{-1} with a measured induction rate of 79±8.0% (Table 1). On the other hand, the lowest induction rate of 8.0±2.0% was obtained when IBA was added at concentration of 2.0 mg L^{-1} to MS medium. No callus was obtained until 8-week when the medium was treated with 2,4-D alone.

Generally, there was a significant difference ($P<0.05$) between NAA and other auxins and no obvious difference was observed between IAA and IBA in their treatments. IAA and IBA treatments produced shoots and root-like structures directly from the explants and then developed callus, where the cells began to undifferentiate at the bottom edges of the explants and then spread throughout. In spite of the differences of the explants sources and plant species, similar results were obtained.[30,31] Calli's morphological remarks were almost similar and no obvious differences were observed (Table 2). Roughly, the callus remarks were not influenced by the PGRs except in the case of 2,4-D calli (Table 2).

The results presented in Table 1 also compared the influence of carbon source concentration on callus induction rate of *S. hermonthica* cultured in the MS media treated with these aforementioned auxins. This study tested the effects of 1.5 and 3% sucrose concentrations on callus induction rate of *S. hermonthica*. The 3% sucrose concentration showed a higher callus induction rate than 1.5% sucrose concentration. Taking into account the differences in the explants source between this study and the previous study, this result is inconsistent with Rousset *et al*., who stated that the callus during sub-culturing gives best growth in 1% and 2% sucrose comparing to higher sucrose concentrations including 3%.[17] These results indicate that the sucrose concentration affect the callus induction of *S. hermonthica*. Although sucrose is the most widely used carbohydrate and carbon source in tissue culture, some reports mention that it may cause hypoxia and ethanol accumulation due to fast metabolism.[31] In our work the increased sucrose concentration was effective for increasing not only the callus induction but also the *in vitro* shoot and root-like structures development of *S. hermonthica* at least at 3.0% sucrose, however, Rousset *et al*., noticed an increasing toxicity of sucrose above 2.0%.[17] Our results were consistent with previous study that reported the importance of sucrose as a prerequisite factor in *in vitro* culture of *S. hermonthica*.[24]

According to the obtained results during this study, regardless of the auxins and carbon source concentrations the superior auxin was NAA. Similar effect of NAA in callus induction for *Orobanche minor* was obtained.[16] All auxins initiated callus at different concentrations except that of 2,4-D, which induced callus only when combined with cytokinin, BAP at 0.5 mg L^{-1}. The organogenesis response of NAA, IAA and IBA calli might be due to the variation of exogenous growth regulators (auxins/cytokinin) and the endogenous growth regulators levels and their balances. The significant differences between the various plant growth regulators alone or in combination with cytokinin indicated that the callus induction of *S. hermonthica* is influenced by the type of growth regulators when used alone or in combination with cytokinin and the sucrose concentration as well. Moreover, the addition of appropriate growth regulator concentration in the medium plays an important role in the callus induction of *S. hermonthica*.

Influence of BAP when combined with auxins at different sucrose concentrations

The BAP cytokinin was combined at concentration of 0.5 mg L^{-1} with different auxins in different concentrations in order to investigate the combination effects on the health and induction rate of the Striga callus. The results presented in Figure 2, showed a high significant difference ($P<0.05$) of the combination with NAA and others auxins in terms of callus induction. When the media were supplied with 3.0% sucrose and 0.5 mg L^{-1} NAA, the highest induction rate of 58±6.0% was obtained. On the other hand, the 2,4-D auxin successfully induced callusing when the MS medium was treated with BAP 0.5 mg L^{-1} combined with 2,4-D, but no response was observed at concentration 4.0 mg L^{-1} 2,4-D. 2,4-D is more likely to work better with BAP at low concentrations than working alone concerning the *S. hermonthica* callus. A previous study suggested similar action for 2,4-D in inducing callogenesis in different genus.[32] IAA and IBA auxins were

Table 1. Effect of auxins alone and sucrose concentration on *S. hermonthica* callus induction.

Sucrose (%)	PGRs	PGRs level vs Callus induction rate (%)				
		0.5	1.0	1.5	2.0	4.0
1.5	NAA	36±2^{a}	42±5^{b}	49±4^{a}	79±8^{b}	30±2^{a}
3.0		34±3^{a}	27±2^{a}	29±3^{a}	22±1^{a}	32±1^{b}
1.5	2,4-D	0.0	0.0	0.0	0.0	0.0
3.0		0.0	0.0	0.0	0.0	0.0
1.5	IAA	10±2^{c}	13±2^{c}	8±2^{c}	12±2^{c}	16±2^{b}
3.0		18±4^{b}	20±3^{b}	24±2^{b}	21±1^{a}	22±1^{a}
1.5	IBA	3±2^{d}	8±2e	17±2^{d}	20±2^{d}	9±2^{c}
3.0		16±3^{b}	12±2^{c}	14±4^{c}	13±2^{c}	17±2^{a}

^{a,b,c,d} values represent Mean ± SEM; means on the same column followed by the same letter are not significantly different at 5.0% level using Duncan's multiple range tests.

Table 2. Summarizes the *S. hermonthica* callus remarks in different auxin treatments on MS medium.

Auxins	1.5% sucrose	3% sucrose
NAA	Pale /yellow green, friable, organogenic	Yellow green, friable, organogenic
2,4-D	Yellow brown, compact/hard, non-organogenic	Yellow, compact/hard, non-organogenic
IAA	Pale brown/yellow, friable, organogenic	Pale brown, friable, organogenic
IBA	Brown/yellow, friable, organogenic	Brown, friable, organogenic

Data were recorded and documented according to the observations on calli remarks on the relevant treatment in week four.

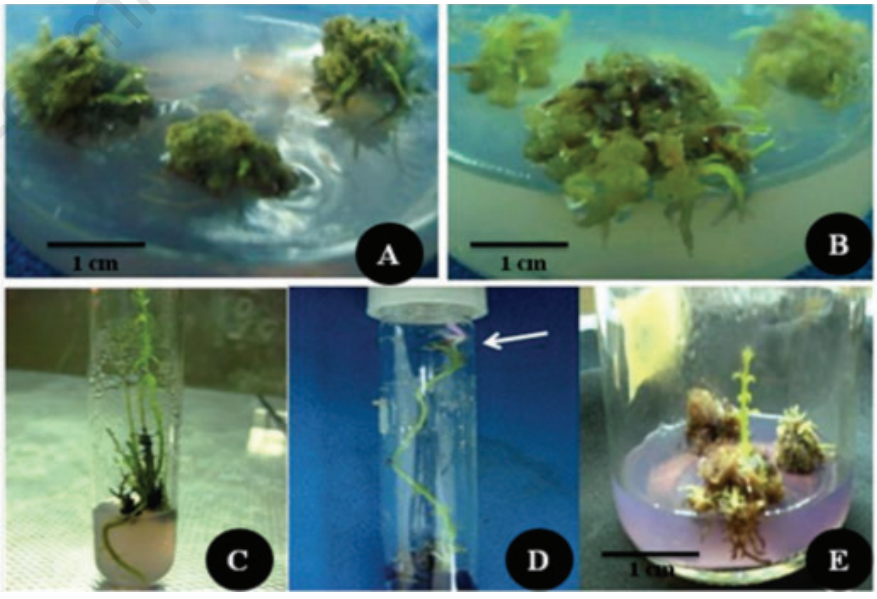

Figure 1. *S. hermonthica in vitro* culture growth response to growth regulators treatments, (A) Calli of MS supplemented with 1.5% sucrose and NAA 1.5%; (B) calli of MS supplemented with 3.0% sucrose and treated with 1.5% NAA; (C) Multiple shoots and root-like structures regeneration from *S. hermonthica in vitro* stem segments cultured on MS basal media, (D) *In vitro* flowering of 8-weeks *S. hermonthica* seedling stem segments cultured on MS basal media; (E) Violet pigments in the MS media secreted by *S. hermonthica* callus, also explains the callus organogenesis activity in response to NAA.

induced callogenesis in slightly lower rate than what they do when used alone. Nevertheless, the callus induction rate seems to be very low in both cases when compared to NAA. The calli's morphological remarks of the combination with NAA, IAA and IBA auxins are almost similar comparing to that obtained when we used auxins alone. In case of combination with 2,4-D the calli's morphological remarks were yellow in color, compact and non organogenic (Table 2).

The results also revealed differences between 0.5 mg L^{-1} BAP in combination with various auxins in MS medium supplemented with 1.5% sucrose, in which 1.0 mg L^{-1} NAA gave best callus induction rate of 56%. However, we noticed no clear differences among different auxins when combined with BAP at different sucrose concentrations, where the 3.0% sucrose was slightly better than 1.5% sucrose with few exceptions that the treatments of 1.0 mg L^{-1} NAA, 2.0 mg L^{-1} and 4.0 mg L^{-1} at 1.5% sucrose had slightly higher induction rates than their corresponding treatments at 3.0% sucrose. As well as the experiments of auxins alone, NAA, IAA, and IBA were regenerated shoot and root-like protrusions after 4-week culture (Figure 1E). However, the very obvious difference at ($P<0.05$) between NAA in one side and 2,4-D, IAA and IBA on the other side, we noticed no difference among 2,4-D, IAA and IBA auxins in the callus induction. It's more likely that the combination of at least 0.5 mg L^{-1} BAP with these auxins do not have strong influence on *Striga* callus induction, except in the case of 2,4-D. The results presented here clearly support our conclusion that the NAA auxin is more likely to be the most effective auxin in an axenic culture of *S. hermonthica*. Nag and Ram reported similar results showing the effect of the auxin growth regulators and its combination with cytokinin on callus initiation and shoot development of the parasitic plant *Dendrophthoe* spp.[33]

Multiple shoots and root-like protrusions were obtained in some sub-cultures of seedling's stem segments cultured in MS basal media during the first two weeks of culture as shown in Figure 1C. These structures were also regenerated in media contained NAA, IAA or IBA at various concentrations (Figure 1A,E). The organogenesis features were not observed when the media was treated with 2,4-D at least not before eight weeks. Multiple shoots were not obtained in media treated with IAA or IBA at 4.0 mg L^{-1} in combination with BAP 0.5 mg L^{-1} up to the 4-week, indicated that IAA and IBA require low concentration when combine with cytokinin for both callogenesis and shoot regeneration purposes. The extreme levels of these auxins might have interfered with the totipotency ability of the stem tissues. In accordance to previous reports,[24,34] some sub-cultured regenerated plantlets of *S. hermonthica* bloomed flowers with light purple color only when sub-cultured in MS basal medium for more than two months, as shown in Figure 1B. Regardless of the auxin/cytokinin levels in the treatments, pale violet and blue pigments were observed in the culture media (Figure 1E). The secretions were observed to start appearing at the end of the second week and the color density was increasing during the long incubation periods. These pigments are probably phenolic compounds according to our preliminary screening and might be responsible for the allelopathic effects of this parasite against its hosts as it has been suggested previously that the presence of high levels of oxidizable phenols and oxidative enzymes detected in the *S. hermonthica* shoot extracts may have a role in the allelophathic activity of this plant.[10,11,35]

Phytochemical screening

Striga hermonthica, causes devastating effects and yield losses on the main cereal crops, which threatens the food security in the developing countries and hitherto the efforts to overcome this hurdles encounter many difficulties. One of the challenges for a selective control of *S. hermonthica* is identification of a metabolic process specific and essential to the parasite that may represent a biochemical target.[17] Understanding the comparative metabolomics of the host and the parasite beside the host-parasite interaction will substantially

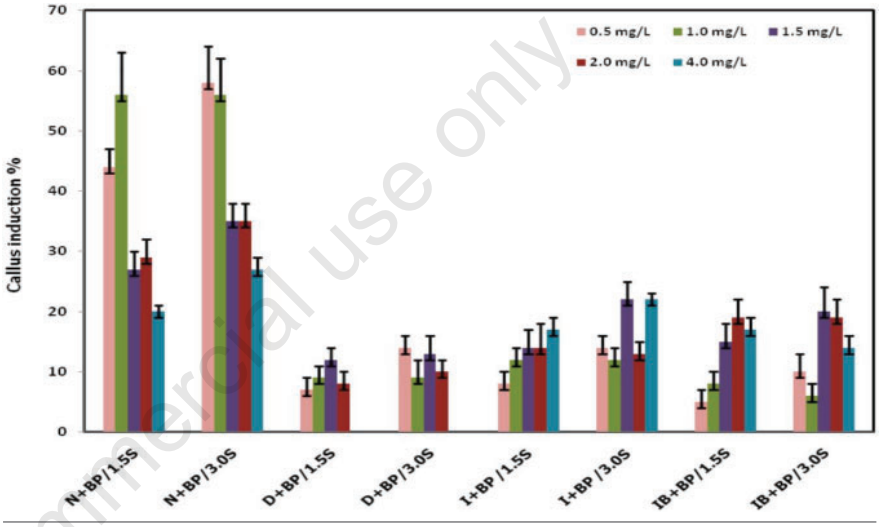

Figure 2. Influence of 0.5 mg/L BAP (cytokinin) when combined with different auxins at different sucrose concentrations on *S. hermonthica* callus induction. Vertical bars represent the Standard Error of Mean (SEM). N = NAA, BP = BAP, D = 2,4-D, I = IAA, IB = IBA, 1.5S = 1.5% sucrose, 3.0S = 3.0% sucrose.

Table 3. Preliminary phytochemical screening of *S. hermonthica* intact plant and callus.

Phytochemical constituents	Screened plant parts Aerial part	Underground parts	Callus
Triterpenes/sterols	++	+	+
Tannins	+	±	±
Coumarins	–	+	+
Cardiac glycosides	+	+	±
Flavonoids	++	+	++
Saponins	++	+	+
Anthracenosides	–	±	–
Emodols	–	–	–
Carotenoids	±	–	–
Glycosides	+	+	+
Reducing compounds	+	+	+
Alkaloids			
Wagner's test			
Mayer's test			

++, reasonable amount, +, moderate amount, ±, trace amount, –, not detected. These amount remarks estimated by observing the test color intensity which was used as indicative sign for the phytochemical quantity.

raise the hope towards overcoming the mystery of its parasitism. Moreover, this parasitic plant has been used extensively in the folkloric medicine, however, little is known about the active secondary metabolites involve in its pharmacological activities.[8-11] The *in vitro* culture system represents a good candidate for such studies.[16] Here, we preliminarily analyzed the phytochemicals of the intact plant and the callus as shown in Table 3. To some extent our results for both intact plant and callus were consistent with Okpako and Ajaiyeoba, who reported the presence of saponins, tannins, flavonoids, volatile oils and cardiac glycosides in the whole intact plants.[10,11] Bell-Lelong *et al.*, were reported high levels of oxidizable phenols and oxidative compounds in the shoot extracts of *S. hermonthica*, which is supported our results that revealed high flavonoids content in the shoot and callus extracts, but a little bit low level in the haustorial part.[35] In addition to these samples, and due to our observation for the unusual secretion of pale purple to drak pigments in the growth medium, we very preliminarily analyzed its constituent, which was likely to be phenolic compounds. These data also support the extensive usages of this plant in the traditional medicine of Africa.[9-11,36]

The present contribution is an attempt to produce a fast growing and healthy calli by using *in vitro* seedling's stem segments as explants. More experiments on testing the combinations of auxins with cytokinins and other growth regulators need to be done to further enhance the callus induction of *S. hermonthica* with high growth rate. The organogenic calli obtained during this study could be a promising candidate as a model for investigating the parasite infection in *in vitro* system. The anatomical structures of the root-like structure as well as the phytochemical constituents of the secreted pigments need to be investigated comprehensively.

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