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Enhancing the *in vitro* development of root hairs for the production of sorgoleone in sorghum

***P. Amali[1], B. Shruthi[1], S. J. Kingsley[2] and S. Ignacimuthu[3]**

[1]*Faculty of Bio & Chemical Engineering, Department of Biotechnology, Sathyabama University, Chennai, India*
[2]*Madras Christian College, Chennai, India*
[3]*Entomology Research Institute, Loyola College, Chennai, India*

ABSTRACT

Sorgoleone exudated from the root hairs of sorghum (Sorghum bicolor (L.) Moench) is a potent bioherbicide. The living root hairs of young developing sorghum plants exude higher content of sorgoleone. The concentration of sorgoleone is proportional to the formation of root hairs. A set of factors that enhance root hair formation and subsequently sorgoleone production were studied under in vitro conditions. The effect of auxins on root growth and root hair formation were studied by incorporating IAA, IBA, NAA and 2,4-D in the concentration range of 0.5- 2.0 mg/L respectively, as a supplement in half-strength Murashige and Skoog (MS) medium. In addition, the effect of gibberellic acid (GA_3) was also studied by supplementing it in half-strength MS medium at a concentration range of 0.5-2.0 mg/L. Among the auxins tested for their in vitro rhizogenic potential, the optimal response was observed in IBA at 1.0 mg/L. Further, GA_3 at 1.0 mg/L also showed a significant optimal effect on root hair formation. The effect of strength of MS medium, addition of copper, phloroglucinol, polyamines such as spermidine and putrescine on optimal root hair induction were also investigated in separate experiments. The results indicated that root hair formation was beneficially promoted in half- strength MS medium containing 1.0 mg/L of IBA and GA_3 respectively. Also inclusion of copper sulfate at 0.5 mg/L in half-strength MS medium in the presence of 1.0 mg/L of IBA and GA_3 respectively, resulted in enhanced root growth and root hair formation. The individual addition of phloroglucinol, spermidine and putrescine to root induction media did not provide any additive effects on promoting root hair formation but however, it was observed that a combination of optimal phytohormones together with copper sulphate and phloroglucinol improved the root hair formation to a significant extent. Further, the optimal temperature for root hair formation was identified to be 30ºC and the roots of 7-days old seedlings showed an increased synthesis of root hairs. An aqueous root extract containing sorgoleone showed bioherbicidal activity against Acalypha indica during in vitro phytotoxic assay.

Key words: Auxins**,** bioherbicide, copper, GA_3, *in vitro*, root hair, sorgoleone.

INTRODUCTION

Sorghum bicolor is an annual crop cultivated for its grain in arid and semi-arid tropical regions of Asia, Eastern Africa and Latin America. Its wider adaptability to heat and drought, commercial usage as food, forage and as a bio-energy crop has rendered its cultivation to over 50 million ha of land yielding a global production of 60 million tons in 2010 [1]. Besides, its allelopathic effect enables its use as a cover crop or as a green manure to suppress weed

populations in agricultural and cropping systems [2]. The prime source for the allelopathic properties of sorghum is due to sorgoleone, an oily exudate from the root hairs of sorghum. Chemically, sorgoleone is 2-hydroxy-5-methoxy-3-[(Z,Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone (molecular weight=358). The synthesis of sorgoleone in root hairs is constitutive and is proportional to the root biomass [3]. Sorgoleone is exuded along with various congeners that include an array of lipid quinones and resorcinols and these congeners differ in the length or degree of saturation of the aliphatic side chain and in the substitution pattern of the aromatic ring [4-6].

The phytotoxic activity of sorgoleone is strongest on small-seeded weeds [5, 7] and broad leaf species are more susceptible to the herbicidal activity of sorgoleone than the grass weed species [8]. The mode of action tends to be the inhibition of photosynthesis by binding to photosystem II (PSII) in lower plants and germinating seedlings and thereby preventing nutrient and water uptake. The selective inhibition of weeds by sorgoleone is due to the fact that it is not translocated acropetally in older plants, but can be absorbed through the hypocotyl and cotyledonary tissues [9]. The effectiveness of sorgoleone as a natural herbicide was found to be comparable to that of many synthetic herbicides such as diuron, atrazine and metribuzin that inhibit photosynthesis [10, 11].

Sorgoleone is produced by the living root hairs of sorghum [12]. Root hairs increase the contact between the plant and the rhizosphere and play a vital role in the absorption of nutrients and water from the soil [13]. Root hair formation and sorgoleone biosynthesis are influenced by various environmental factors such as moisture [14], temperature, light, pH and external stimulus such as velvetleaf root extract [3]. Increasing the root hair formation would proportionally increase the yield of sorgoleone. The current study was conducted to determine the factors that enhance root growth and sorgoleone production under *in vitro* conditions and to determine the phytotoxic activity of sorgoleone root extract. This would enable the mass production of sorgoleone and its subsequent development as a bioherbicide.

MATERIALS AND METHODS

Plant material and growth conditions:
Seeds of *Sorghum bicolor* procured from Tamilnadu Agricultural University, Coimbatore, were used to initiate the study. The seeds were soaked for about 2-3 hours and they were then washed in 50 µl of diluted tween-20 for 5 minutes. The detergent solution was removed by rinsing the seeds thoroughly in distilled water. Further surface sterilization procedures were carried out in the laminar air-flow chamber. For this, the seeds were treated with 70% ethanol for 30 seconds and subsequently with 0.1% mercuric chloride for 5 minutes. The seeds were then rinsed with sterile distilled water for 5 times to remove the surface sterilants and were dried in sterile Whatman #1 filter papers prior to inoculation in half-strength MS medium [15] solidified with 0.6% (w/v) agar. The cultures were maintained under dark incubation. A set of external factors were analyzed in the consecutive experiments to optimize the formation of root hairs for the extraction of sorgoleone.

Effect of auxins and GA_3 on root growth and root hair formation:
To determine the optimal auxin and its concentration for root growth and root hair formation, sorghum seeds were inoculated in half-strength MS media containing IAA, IBA, NAA and 2,4-D respectively, at a concentration range of 0.5-2.0 mg/L. Similarly, GA_3 (0.5- 2.0 mg/L) was also supplemented in half-strength MS media to analyze its effect on root hair induction. A control containing seeds in half-strength MS basal medium was also prepared. The cultures were maintained at 28±2ºC in dark for about 7 days. The root length and the percentage of root hair formation were calculated on the 7^{th} day of inoculation. Each experiment included around 20 seeds and the root hairs were observed under the light microscope at 40X magnification.

Effect of strength of MS medium on root growth and root hair formation:
The sorghum seeds were inoculated in full-strength and half-strength MS medium containing the optimal concentration of auxin and GA_3 respectively, to determine the optimal medium strength for root hair induction. The root growth and frequency of root hair formation (%) were studied using the 7^{th} day-old-seedlings.

Effect of temperature on root hair formation:
In a separate experiment, to study the influence of temperature on root hair formation, two sets of flasks each containing 20 seeds were inoculated in half-strength MS medium containing the optimal concentration of auxin and GA_3 respectively and each set was incubated at two different temperatures of 28ºC and 30ºC respectively. The results were recorded on the 7^{th} day after inoculation.

Effect of age of seedlings on root hair formation:
Since the young developing plants are known to exude higher content of sorgoleone, the effect of age of *in vitro*-grown seedlings was evaluated for the maximum root hair synthesis. Consequently, root hair formation in 5 days, 7 days and 10-days-old-seedlings were studied and the data were collected.

Effect of Copper and phloroglucinol on root hair formation:
Copper in the form of copper sulfate at 0.5 mg/L and Phloroglucinol at 0.1 mg/L were included both separately and together in combination, in the root induction medium containing optimal concentrations of phytohormones in half-strength MS medium, to evaluate their enhancing effect on *in vitro* rhizogenesis.

Effect of polyamines on root hair formation:
The effect of addition of polyamines such as spermidine and putrescine at 0.1 mg/L concentration each, to the optimal root induction media were also investigated to identify their role in *in vitro* root hair development.

Extraction and sorgoleone analysis by GC-MS:
The extraction protocol included methanol as the solvent and roots from 7-days-old sorghum seedlings were immersed in methanol (1:20 w/v) for 30 sec to obtain a crude extract. It was filtered and evaporated under vacuum to obtain a dried powder. The dried extract was then redissolved in methanol (1mg/ml) and subjected to GC-MS analysis.

***In vitro* phytotoxic assay:**
To test the efficacy of sorgoleone as a potent bioherbicide, an *in vitro* assay was performed using the fresh leaves of a herbaceous weed, *Acalypha indica.* The fresh green leaves were cleaned, surface sterilized using 0.1% mercuric chloride for 10 minutes, washed 3 times in sterile distilled water and inoculated onto petridishes containing autoclaved MS basal medium. To these leaf cultures, around 1ml of aqueous extract containing sorgoleone was added and incubated at 28±2°C in dark conditions along with control. The results were recorded after 3 days of inoculation.

RESULTS AND DISCUSSION

The sorghum seedlings germinated after 3 days of inoculation in the dark and the radicle development was observed in half-strength MS medium containing the respective auxins at defined concentrations. Among the auxins studied, the optimal root growth and root hair formation was observed in IBA at 1.0 mg/L with dense root hairs on the 7^{th} day of inoculation (Table. 1; Fig. 1A-F). Several studies have reported IBA as the optimal rooting hormone for *in vitro* regeneration in sorghum [16, 17]. Further, root growth with root hair formation was also pronounced at 2.0 mg/L of IAA and at 0.5 mg/L of NAA. It was found that NAA produced stunted roots with dense root hairs. However, root growth was hindered by callus induction with increasing concentrations of NAA and with 2,4-D. Several reports are available pertaining to the predominating role of 2,4-D as a callus inducing hormone in cereals [16-19]. Further, GA_3 at a concentration of 1.0 mg/L was also found to be optimal with a higher proportion of root hairs. GA_3 is known to induce seed germination and shoot elongation by increasing cell division rather its effect on *in vitro* rooting is uncertain [20]. However, our results have shown that GA_3 can promote root initiation and elongation along with dense root hairs (Fig. 1G).

The half-strength MS medium was found to be more beneficiary in promoting root induction and subsequently root hair formation, whereas the full-strength medium showed a minimal root growth in presence of optimal phytohormone concentrations (Table. 2). Also it was observed that temperature influenced the root hair formation. Thus at two different temperatures 28ºC and 30ºC, the root length and root hair formation varied wherein a higher frequency of root hair formation was favored at 30ºC, marking it to be optimal (Fig. 1E&H). An earlier study also has reported 30ºC as the optimum temperature for root growth and sorgoleone production [3]. Sorgoleone production decreases with the age of seedlings as young developing plants are known to exude higher contents of sorgoleone. Among the seedlings analyzed for root length and root hair formation on 5^{th}, 7^{th} and 10^{th} days, the 7-days-old seedlings proved to be optimal in root hair formation (Table. 3). However, the root length increased in 10-days-old seedlings but with less frequency of root hairs. This was due to the formation of successive secondary and tertiary roots from the primary roots that declined the root hair formation.

Table 1. Effect of auxins and GA_3 on root growth and root hair formation

Half-strength MS medium + PGRs	Concentration in (mg/L)	Mean root length (cm)	Frequency of root hair formation (%)
Control	-	2.6	10
IAA	0.5	2.5	30
	1.0	3.2	50
	2.0	5.4	80
IBA	0.5	2.9	40
	1.0	6.2	90
	2.0	3.7	70
NAA	0.5	1.6	75
	1.0	0.8	50
	2.0	1.4	10
2,4-D	0.5	1.2	15
	1.0	1.0	5
	2.0	0.4	0
GA_3	0.5	3.2	75
	1.0	5.9	85
	2.0	4.6	60

Table 2. Effect of strength of MS medium and temperature on root growth and root hair formation

Strength of MS medium	Temperature	PGRs	Concentration in (mg/L)	Mean root length (cm)	Frequency of root hair formation (%)
Half-strength	28ºC	IBA	1.0	7.5	85
		GA_3	1.0	4.7	80
Full-strength	28ºC	IBA	1.0	4.4	10
		GA_3	1.0	2.4	15
Half-strength	28ºC	IBA	1.0	6.6	80
		GA_3	1.0	5.1	80
Half-strength	30ºC	IBA	1.0	7.1	95
		GA_3	1.0	6.4	90

Table 3. Effect of age of seedlings on root growth and root hair formation

Half-strength MS + PGRs	Concentration in (mg/L)	Mean root length (cm)			Frequency of root hair formation (%)		
		5^{th} day	7^{th} day	10^{th} day	5^{th} day	7^{th} day	10^{th} day
IAA	2.0	4.7	5.8	7.4	40	80	75
IBA	1.0	6.8	7.3	9.4	70	85	80
NAA	0.5	2.1	2.6	3.7	60	70	70
GA_3	1.0	4.9	6.8	8.3	70	90	70

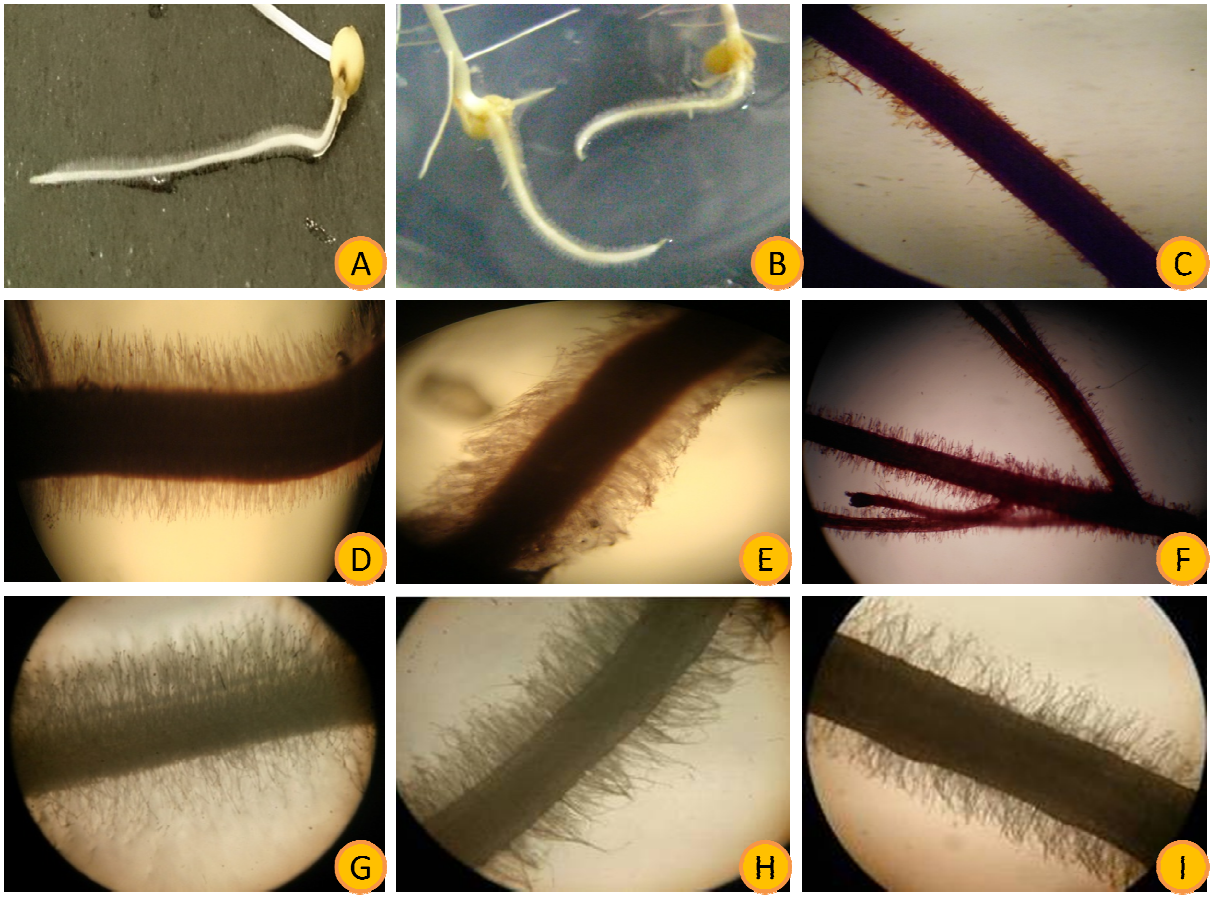
Table 4. Effect of copper, phloroglucinol and polyamines on root growth and root hair formation

Elicitor molecules	Half-strength MS + PGRs	Concentration of PGRs (mg/L)	Mean root length (cm)	Frequency of root hair formation (%)
$CuSO_4$	IBA	1.0	11.4	90
	GA_3	1.0	9.8	85
Phloroglucinol	IBA	1.0	6.9	80
	GA_3	1.0	6.6	80
$CuSO_4$ + phloroglucinol	IBA	1.0	12.7	90
	GA_3	1.0	10.1	90
Spermidine	IBA	1.0	5.8	30
	GA_3	1.0	4.3	10
Putrescine	IBA	1.0	3.9	40
	GA_3	1.0	5.3	15

Copper is an essential micronutrient that supports the *in vitro* growth and differentiation of cultured plant tissues. It plays vital role as a cofactor for many enzymes in electron transport, chloroplast, protein and carbohydrate biosynthesis and polyphenol metabolism [21, 22]. The addition of copper as copper sulphate at 1µmol/L in root induction medium had significantly increased root proliferation from immature embryos of sorghum [23]. Similarly, our experiments with 0.5 mg/L of copper sulphate improved the root proliferation with elongated roots containing thick dense root hairs (Table. 4). Phloroglucinol stimulates rooting by acting as auxin synergists or auxin protectors [24]. Very less increase in root growth with declined root hair frequency was observed with the inclusion of

phloroglucinol in root induction medium, but however, phloroglucinol in combination with copper sulphate was found to be effective in increasing the root growth and root hair formation (Fig. 1I). There are reports stating the stimulatory effect of polyamines on *in vitro* rooting [25, 26]. In sorghum, the regeneration response to the concentration of polyamines was observed to be genotype-specific [27]. In our study, the addition of spermidine or putrescine to root induction medium resulted in a decline in the root growth and root hair formation.

Figure 1. *In vitro* formation of root hairs for sorgoleone production in sorghum



Root hairs from 7-days-old seedlings on half-strength MS medium supplemented with IBA at 1.0 mg/L (A&B), microscopic observation of root hairs on half-strength MS medium (C), supplemented with IBA at 1.0 mg/L (D), incubated at 30°C (E) and secondary roots showing root hairs (F), root hairs in half-strength MS medium containing GA_3 at 1.0 mg/L (G), incubated at 30°C (H) and root hairs in a combination of 1.0 mg/L IBA + 0.5 mg/L $CuSO_4$ + 0.1 mg/L phloroglucinol (I).

The extraction procedure for sorgoleone was initially performed by immersing the roots of sorghum in methylene chloride and 1% glacial acetic acid [28]. However, methanol was also reported to be a good solvent for sorgoleone extraction [29]. The analysis of methanolic root extract of sorghum by GC-MS revealed the presence of fatty acids, benzoquinone and structurally related hydroquinones (Fig. 2)

During the *in vitro* assay, an aqueous extract of sorgoleone added to the cultured leaves of *Acalypha indica* induced chlorosis and browning of leaves, after 3 days of inoculation indicating a phytotoxic activity while the control plates were observed to be photosynthetically active (Fig. 3A&B). This is an evidence of the potential phytotoxic effect and herbicidal activity of sorgoleone that is associated with the inhibition of photosystem II (PSII) [30]. Sorgoleone interferes with the photosynthetic machinery and mitochondrial electron transport by mimicking the natural electron acceptors plastoquinones and ubiquinone, respectively [31-34] and also inhibits the activity of p-hydroxyphenylpyruvate dioxygenase, a key enzyme in plastoquinone biosynthesis [35].

Figure 2. GC-MS analysis of root extract for sorgoleone synthesis

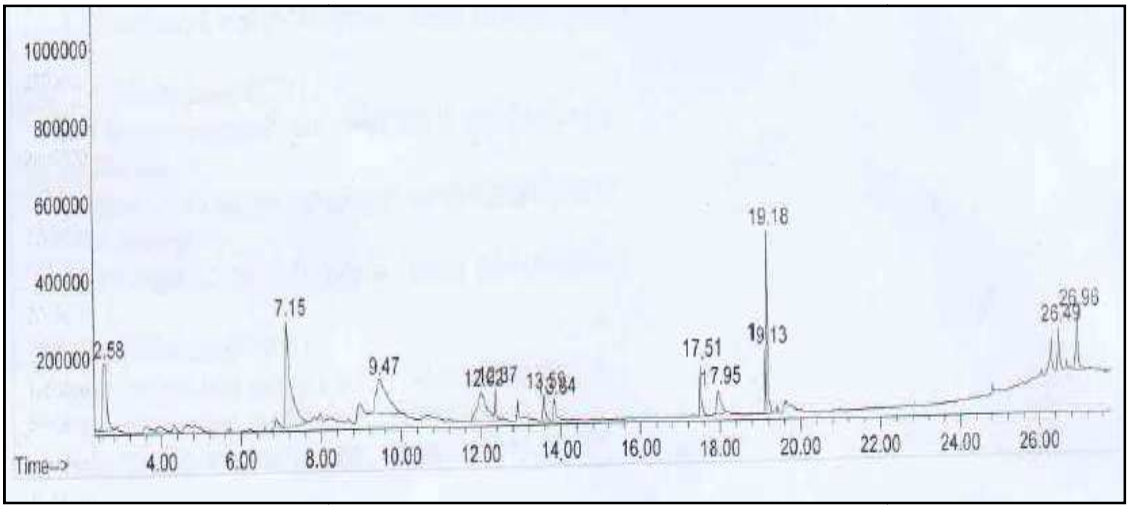
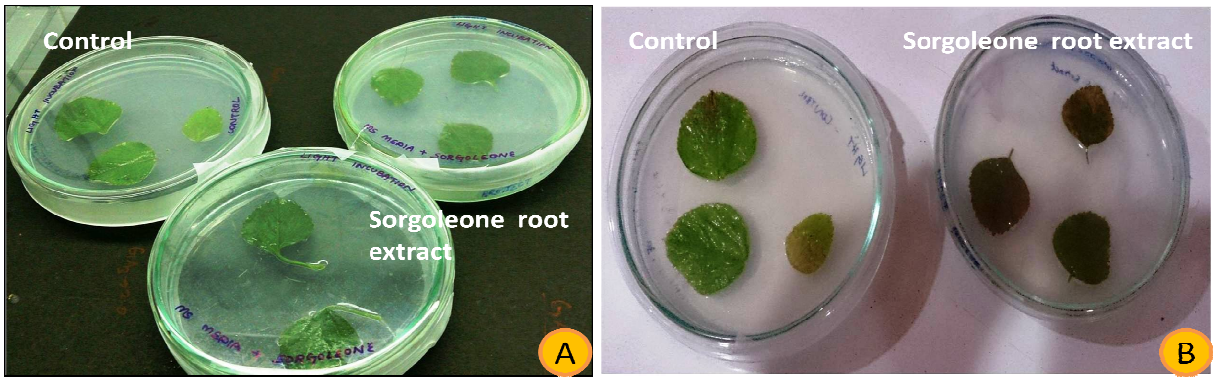


Figure 3. *In vitro* phytotoxic assay using sorgoleone root extract in *Acalypha indica*



A) Leaves after inoculation on MS medium at 28±2°C **B) Leaves observed after 3 days of inoculation on MS medium**

CONCLUSION

The *in vitro* development of root hairs was significantly increased by supplementing the half-strength MS medium with IBA at 1.0 mg/L and GA_3 at 1.0 mg/L respectively. Further, temperature and the age of seedlings also influenced the root hair synthesis and the addition of elicitors such as copper, copper + phloroglucinol to the optimal root induction media improved the *in vitro* formation of root hairs and this *in vitro* enhancement protocol could be applied for the mass production of sorgoleone from sorghum root hairs, to enable its effective use as a commercial bioherbicide.

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