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Microrhizome induction in *Kaempferia galanga* L. using silver nanoparticles

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Abstract

Kaempferia galanga L. is an economically important medicinal plant used in several ayurvedic formulations and over-exploited to the extent that there is always scarcity of rhizomes that are the propagating material. The present study takes into account of the beneficial role of silver nanoparticles (AgNPs) on microrhizome production in this taxa. The concentration of AgNPs were varied (such as 12.5, 25, 50, 100 and 200 mgl^{-1}) in the Murashige and Skoog (MS) nutrient medium supplemented with 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA and the different treatments were designated as *T1*, *T2*, *T3*, *T4* and *T5*. Variable morphological features with respect to shoots and roots were noticed in the treatments compared to control. Comparatively short shoots were noticed in AgNP-treated plants. Roots were tuberous that intrinsically varied in their morphology in different concentrations of AgNPs. Induction of microrhizome was seen in all AgNP treatments irrespective of its concentration and it was at maximum intensity in *T2* (25 mgl^{-1} AgNPs). The synergistic effect of AgNP-treatment along with 6% (w/v) sucrose supplementation and dark period incubation hastened the partitioning of assimilates to the storage parts thereby causing the development of microrhizomes in *K. galanga*. The induction of microrhizoes was confirmed by the anatomical studies and it was compared with the rhizomes formed in the field (mother rhizomes). The outcome of this study can be further used for mass production of pathogen-free microrhizomes and conservation of *K. galanga* for its sustainable utilization.

Keywords: *Kaempferia galanga*, microrhizomes, AgNPs, sucrose, photoperiod, anatomy, conservation

Introduction

In Zingiberaceae, biotechnological interventions especially *in vitro* culture techniques provide an alternative means of propagation and tool for crop improvement. Conventionally in zingibers, the rhizomes are used as planting materials which encounter several disadvantages such as low multiplication rate by rhizome separation method, requirement of large amount of rhizomes for propagation in the next season and senescence and degradation due to intrinsic pathogens and disease incidence in field and storage. These disadvantages led to the realisation of the utmost importance of *in vitro* microrhizome induction in zingibers. The microrhizomes resemble the normal rhizome in all respects, except for their smaller size, their storage and transport are also easier, thus facilitating germplasm exchange. Also, these can be moved to the field directly, without acclimatization or hardening. Microrhizomes of numerous rhizomatous plants were found to develop *in vitro* with high sucrose levels, they can be effortlessly transported and bigger microrhizomes were fit for endurance in the field without any acclimatization method [1]. The targeted plant material of the present study *Kaempferia galanga* L., a Zingiberaceae species, is dispersed all through the plains of India and displays poor natural regeneration by seeds or rhizomes, in this way arriving at the edge of being perceived as an endangered species with potent restorative capacity[2]. The plant is overexploited and listed under threatened category in India, Sri Lanka and many Asian Countries [3]. The leaves, rhizome and root tubers of the species have various medicinal applications. It is economically significant and is over used to the degree that there is consistently shortage of propagating material (rhizomes) which is the consumable part as well. *K. galanga* is utilized as spice, condiment, medication and in beauty care products [4]. In Rheede's *Hortus Malabaricus*, *K. galanga* L. has been portrayed under the name 'katsjula kelengu' which shows that the plant was utilized as a medication source in Kerala in the 17th century [5]. It forms a component of over 59 ayurvedic medicines [6] and is extensively used in preparation of herbal drugs, perfumery, cosmetics and as spice ingredients [7]. It is utilized for treatment of diarrhoea, headache and it builds vitality to beat weariness [8]. The rhizomes and root stocks are bitter, thermogenic, acrid, carminative, aromatic, depurative, diuretic, expectorant, digestive, vulnerary, anthelmintic, febrifuge and stimulant. They are good for dyspepsia, leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, wounds, ulcers, helminthiasis, fever, malaria, splenopathy, inflammatory tumor, nasal obstruction and hemorrhoids [4]. As folk medicine, the rhizomes are employed for antibacterial, treatment of hypertension, asthma, rheumatism, indigestion, cold and headache, relief abdominal pain and toothache [9]. The powdered rhizome mixed with honey is an expectorant used to treat cough and pectoral affection. Besides, oil prepared from the rhizome is applied over the nasal region to relieve nasal congestion [10]. It has been reported recently

that the rhizomes contains the chemicals that are of insecticidal potent [11]. Its wound healing activity is also proved [12]. The cytotoxic activity of the crude alcoholic extract and successive extracts of rhizome of this plant in both normal and cancer cell cultures has been reported [13].

Conventional propagation of *K. galanga* is through rhizomes, which remains dormant during drought and sprouts only in spring and there is very poor seed setting naturally. *In vitro* propagation of *K. galanga* through multiple shoot induction and organogenesis has been reported by several authors [2, 14, 15, 2, 16-19, 7, 20- 23, 3]. However, reports on microrhizome induction on this plant are scarce and there is only a single one in this aspect. Though experiments on the effect of silver nitrate on microrhizome induction in turmeric has been carried out [24], no studies yet on the usage of AgNPs in any zingibers and particular in *K. galanga.* Moreover, there are promising reports on the effect of AgNPs in *in vitro* culturing in some plant species [25-29]. The application of silver nanoparticles (AgNPs) has effectively controlled the microbial contaminants from explants [30] and this has demonstrated the positive part of AgNPs in callus induction, organogenesis, somatic embryogenesis, somaclonal variation and secondary metabolite production [31]. Therefore, it is the best interest to study the role of AgNPs in inducing microrhizomes in *K. galanga*. The anatomy of *in vitro* microrhizomes formed in *Curcma longa* [32] and *K. rotunda* [33] were reported earlier. Here we also discussed the anatomy of microrhizomes in comparison with the mother rhizomes of *K. galanga*.

Materials and methods

Plant material

Rhizomes of *Kaempferia galanga* L. collected from the cultivation sites of Kundara, Kollam District of Kerala, India were used as the plant material for the *in vitro* shoot culture establishment and subsequent microrhizome induction experiments in the present study.

Shoot culture establishment

Fresh rhizomes with axillary buds collected from the field-grown plants were thoroughly washed under running tap water, outer scales were removed and washed in 5% Teepol (v/v), for 20 minutes and again washed in running tap water. After several rinses in distilled water, the explants were subjected to sterilization with 0.1% (w/v) $HgCl_2$ for 8-10 minutes followed by 4-5 rinses in sterile distilled water. Rhizome with axillary buds are inoculated aseptically in Murashige and Skoog (MS) medium [34] containing 0.5 mgl^{-1} TDZ. Initiated shoots were subcultured after 4 weeks to fresh MS medium augmented with 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA for shoot multiplication.

Microrhizome induction

To examine the effect of different concentration of AgNPs on *in vitro* microrhizome induction, the *in vitro* de-topped shoot segments having a rhizome base (1 cm) established after 4 weeks in 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA were transferred to the fresh medium of the same PGR composition augmented with different concentration of AgNPs *viz.* 12.5, 25, 50, 100 and 200 mgl^{-1} and 6% (w/v) sucrose. AgNps (Sigma-Aldrich) having 100 nm particle size, at 0.02 $mgml^{-1}$ concentration in aqueous buffer containing sodium citrate as stabilizer was used in the experiment. The different AgNP treatments were designated as *T1*, *T2*, *T3*, *T4* and *T5* respectively.

All the inoculated culture tubes were incubated in a culture room (26±2 ºC) under 8 hour photoperiod at a photon flux intensity of 50-60 $\mu Em^{-2}s^{-1}$ provided by cool, white, fluorescent tubes (Philips, India) under 50-60% RH for the initial four weeks period and then incubated for another four weeks under darkness.

Anatomical studies

Anatomical studies were conducted to confirm the rhizome formation and their development. For that thin transverse sections of mother rhizomes and microrhizomes were taken and stained with Safranine (0.3%) and mounted in glycerine. The amount oil cells were observed under trinocular microscope and photographed.

Statistical analysis

Each treatment consisted of ten replicates and were repeated thrice. The observations on number of shoots and roots, length of shoots and roots, number of leaves per shoot, nature of roots and microrhizome induction were recorded after 8 weeks. Results were presented as mean±SE. Data were subjected to ANOVA and the means were compared by Duncan's multiple range test at $p \leq 0.05$ using the statistical package SPSS (version 10.0).

Results and Discussion

In vitro shoot culture establishment

In vitro shoot cultures were initiated from surface sterilized rhizome explants of *K. galanga* in MS medium containing 0.5 mgl^{-1} TDZ (Fig. 1a). This is in accordance with the early findings in this species [23]. Initiated shoots were multiplied after 4 weeks in fresh MS medium augmented with 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA. Shoot segments isolated from such established shoot cultures (Fig. 1b) were used in subsequent microrhizome induction.

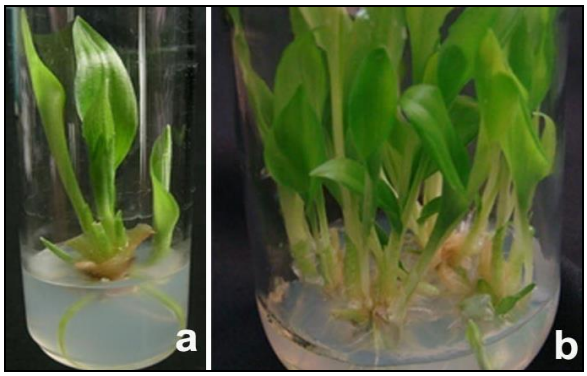

Fig 1: (a) Shoot culture initiation in MS + 0.5 mgl^{-1} TDZ; (**b**) Shoots multiplied in MS+0.1 mgl^{-1} TDZ +2.0 mgl^{-1} NAA.

Microrhizome induction

The partitioning of nutrients and assimilates in different organs and tissues is in a constant state of flux throughout the growth and development of a plant [35] and assimilate partitioning from source to sink is essential for the harvest of economically important plant parts. In *K. galanga*, the rhizomes are the harvestable part of economic importance and they serves as the sink where assimilates are unloaded. Earlier it has been established in this taxa that higher concentration of sucrose (6 or 9%) and high cytokinin supplementation have induced microrhizomes in a normal

photoperiod of 16 hr illumination. This occurred as the assimilate provided for the *in vitro* condition i.e., sucrose might have been transported to the stem for rhizome initiation [18]. There are previous reports on microrhizome induction in many rhizomatous species and their cultivars such as ginger [36-41], turmeric [42-44, 1], *Alpinia* [45] and *Kaempferia* [18, 46]. The present study carried out for the development of a protocol for *in vitro* rhizome induction using AgNPs in *K. galanga* has provided information on the variation in shoot number, shoot length, number of leaves, root number, root length and the nature of the roots due to difference in concentration of AgNPs added to the medium, the effect of these treatments as well as the concentration of sucrose and photoperiod in evoking microrhizome induction in the targeted species.

Morphogenic response of shoots upon treatment with AgNPs

The *in vitro* shoot segments of *K. galanga* transferred to different concentrations of AgNPs exhibited varied responses with regard to the morphological parameters analyzed. The control shoots devoid of AgNPs treatment exhibited multiple shoots (4.50±0.16). However, the multiplication frequency was apparently high in most of the treatments, with an exception in *T2*. Increased number of shoots were noticed in the *T3*, *T4* and *T5* treatments which produced an average of 10-12 multiple shoots (Fig. 2); while the least number of shoots (3.70±0.15) were found in plants grown in *T2* (25 mgl^{-1} AgNPs). In agreement with this, in *Tecomella undulata*, the incorporation of AgNPs in the culture medium positively stimulated the production of multiple shoots. Also, AgNP treatment delayed explant senescence and increased survival by down regulation of the *TuACS* gene (Amino Cyclopropane 1-carboxylic acid synthase gene in *Tecomella undulata*) that is responsible for ethylene synthesis [27]. In the case of the length of shoots, the control plants showed maximum length (7.27±0.07 cm) and upon AgNP-treatments, the shoots were comparatively short (Table 1) (Fig. 2). With regard to the number of leaves per shoot, there was an asynchronous variation noticed. More number of leaves (~14) per shoots were observed in *T3* and *T4* compared to control that produced 7.40±0.16 leaves; while only 5.10±0.10 leaves were recorded in *T2* that contained 25 mgl^{-1} AgNPs (Fig. 2). Overall, the *T5* medium containing 200 mgl^{-1} AgNPs was found to be most favorable for shoot multiplication in *K. galanga*.

Table 1: Morphogenic response of shoots of *K. galanga* upon treatment with AgNPs

Treatments (AgNP Conc.) (mgl^{-1})	Shoot number	Shoot length (cm)	Number of leaves	Microrhizome induction (% response)
Control	4.50 ± 0.16^{d}	7.27 ± 0.07^{a}	7.40 ± 0.16^{d}	NIL
T1 (12.5)	6.70 ± 0.15^{c}	4.49 ± 0.07^{c}	9.80 ± 0.13^{c}	Noticed (100.0%)
T2 (25)	3.70 ± 0.15^{e}	5.17 ± 0.06^{b}	5.10 ± 0.10^{e}	Noticed (93.3%)
T3 (50)	10.80 ± 0.25^{b}	4.55 ± 0.07^{c}	14.10 ± 0.22^{a}	Noticed (96.7%)
T4 (100)	11.60 ± 0.30^{a}	4.55 ± 0.06^{c}	14.80 ± 0.32^{a}	Noticed (96.7%)
T5 (200)	11.80 ± 0.29^{a}	4.70 ± 0.05^{c}	12.60 ± 0.23^{b}	Noticed (96.7%)

*Data represents mean±SE of ten replicates repeated thrice, recorded after 4 weeks of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and Duncan's multiple range test at $p \leq 0.05$.

Fig 2: Deflasked shoots from control and AgNP-treated cultures

Morphogenic response of roots upon treatment with AgNPs

In the present study, maximum number of roots were seen in control plants, and they were fibrous, slender and the number was exceeding 40. Similar root morphotype was reported in the same species during the transfer of microshoots to shoot multiplication medium [18]. Among the different AgNP-treatments, maximum roots were recorded in *T5* (31.70±0.42) and the least number was seen in *T3* (14.60±0.47) and *T1* (14.70±0.21) respectively (Table 2; Fig. 3). Taking into consideration of the root length, elongated slender fibrous roots having a length of 2.55±0.03 cm were seen in the control; while the minimum length (0.86±0.08 cm) was noticed in the *T5* medium (Fig. 3). Regarding the nature of the roots, different AgNP-treatments induced different root morphotypes. The roots of the plants grown in the control were more fibrous and slender whereas those raised in the *T3*, *T4* and *T5* medium were tuberous in nature (Fig. 3). The tuberous roots intrinsically varied in their morphology in different concentrations of AgNPs. More tuberous roots along with some little fibrous roots were noticed in *T1* treatment, while

in *T3*, *T4* and *T5*, the tuberous nature changed to medium, short and very short tuberous types as evident in their number and length as recorded in Table 2. Stout, tuberous, pear-shaped roots typical of microrhizomes were observed in *T2* treatment (Table 2; Fig. 3). Previously, *K. galanga* microshoots cultured in MS medium aided with 6% (w/v) sucrose and either BA (22.20 µM) or Kinetin (23.25 µM) exhibited pear-shaped microrhizomes [18] wherein, the microrhizomes were induced only during the second subculture phase. Thus it is obvious that the AgNP-treatments had a strong influence on inducing microrhizomes in *K. galanga* which can be correlated with the nature of root morphotype. The microrhizome induction was noticed with less intensity in *T1* which exhibited tuberous roots along with few fibrous ones; while it was maximum in *T2* that produced tuberous, pear-shaped roots and then the intensity decreased further in *T3*, *T4* and *T5* (Table 2).

Table 2: Morphogenic response of roots upon treatment with AgNPs

Treatments (AgNP Conc.) (mgl^{-1})	Root number	Root length (cm)	Nature of roots	Intensity of microrhizome Induction
Control	Numerous (>40)^{a}	2.55±0.03^{a}	More fibrous and slender roots	NIL
T1 (12.5)	14.70±0.21^{e}	1.66±0.02^{c}	Less fibrous roots, more tuberous roots	++
T2 (25)	24.60±0.26^{c}	2.12±0.06^{a}	Stout, tuberous, pear-shaped roots	++++
T3 (50)	14.60±0.47^{e}	1.78±0.01^{b}	Medium tuberous roots	+++
T4 (100)	21.40±0.92^{d}	1.31±0.06^{d}	Short tuberous roots	++
T5 (200)	31.70±0.42^{b}	0.86±0.08^{e}	Very short tuberous roots	++

*Data represents mean values of ten replicates repeated thrice, recorded after 4 weeks of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$.

Effect of AgNPs on *in vitro* microrhizome induction *K. galanga*

MS medium augmented with in 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA and different concentration of AgNPs (12.5, 25, 50, 100 and 200 mgl^{-1}) along with 6% (w/v) sucrose were tested for *in vitro* microrhizome induction in *K. galanga*. Fresh weight of the plant, shoot leaf biomass and weight of microrhizomes were noted after three months. Maximum fresh weight of the plant (4.56±0.13g) was detected in the control plant without AgNPs, but it failed to produce *in vitro* microrhizomes. In T_1 fresh weight of the plant was 3.94±0.21g and good amount of *in vitro* microrhizomes was observed in this treatment. Highest amount of microrhizomes (1.81±0.09 g) were generated in T_2 (25 mgl^{-1} AgNPs) and least amount was recorded in T_5. From the results it is clear that AgNPs has a stimulating effect on *in vitro* microrhizome induction in *K. galanga* which may be due to the inhibition of ethylene production as observed in *Tecomella undulata* [47]. Siver nitrate ($AgNO_3$) is a salt of silver and it is commonly used as an anti-ethylene compound in plant tissue culture [48]. Ethylene hormone attaches to its receptors in the presence of copper ions. It has been proved that silver ions could be substituted by copper ions because of similarity in size and thus blocks the receptors and prevent the response from ethylene [49, 50]. Silver nitrate reduces ethylene production by inhibiting amino cyclopropane -1 carboxylic acid, that present is in ethylene biosynthetic pathway [51] thus stimulating microrhizome induction. Induction of microrhizomes visible in all AgNPs-treatments suggest that AgNPs are potent inhibitors of ethylene, acting in a similar way of $AgNO_3$ that act synergistically along with sucrose and thus improving microrhizome induction in *K. galanga*.

Table 3: Effect of AgNPs on microrhizome induction in *K. galangal*

Treatments (AgNP Conc.) (mgl^{-1})	Fresh weight of the plant (g)	Shoot leaf biomass (g)	Weight of microrhizomes (g)
Control	4.56±0.13^{b}	3.56±0.02^{b}	0.52±0.05^{f}
T_1 (12.5)	3.94±0.21^{c}	2.00±0.14^{c}	1.75±0.07^{b}
T_2 (25)	4.85±0.22^{a}	2.53±0.09^{a}	1.81±0.09^{a}
T_3 (50)	3.71±0.09^{d}	1.56±0.20^{e}	1.53±0.01^{c}
T_4 (100)	3.43±0.18^{e}	1.86±0.14^{c}	1.15±0.03^{d}
T_5 (200)	3.24±0.11^{f}	1.79±0.07^{d}	1.08±0.02^{e}

*Data represents mean values of ten replicates repeated thrice, recorded after 4 weeks of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$.

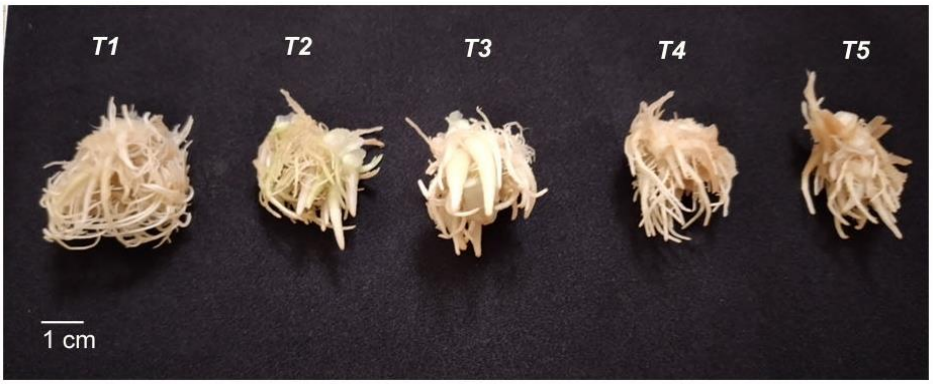

Fig 3: Induction of micro rhizomes and tuberous roots in *K. galanga* upon AgNPs treatment

In the present study, the data on microrhizome induction were recorded after 8 weeks and the cultures were incubated under 8 hr photoperiod for the initial 4 weeks followed by dark period for another 4 weeks. The influence of photoperiod was found to have a significant role in the induction of microrhizome in ginger [52] which is in corroboration with the findings presented here. In *K. galanga*, initially the shoot cultures were raised in MS medium augmented with 0.1 mgl^{-1} TDZ and 2.0 mgl^{-1} NAA and the microrhizomes were induced when the shoot segments from these established cultures were transferred to fresh medium of the same PGR composition augmented with different concentration of AgNPs incubated under 8 hr photoperiod for initial 4 weeks and subsequently in the dark for the next 4 weeks period. In agreement with this, the effect of sucrose and BA in the medium, as well as photoperiod and their interaction in the induction of microrhizomes in turmeric was reported [53]; wherein medium containing 13.3 µM BA and 60 gl^{-1} sucrose with 4 hr photoperiod was found to be most effective for microrhizome induction. Recently, pathogen-free microrhizome production in ginger in MS medium containing 0.5 mgl^{-1} BA and 0.5 mgl^{-1} IAA and 8% (w/v) sucrose under 8 hour photoperiod was also established [54]. Based on these inferences, it can be concluded that microrhizome induction occurred in *K. galanga* due to the synergistic effect of AgNP-treatment and high sucrose concentration along with incubation under dark period. Incubation of the cultures in the dark period during the establishing phase might have stimulated the partitioning of assimilates to the storage organs and this may have evoked the induction and development of microrhizomes more efficiently in *K. galanga.* The induced microrhizomes upon subculture in basal medium aroused the proliferation of axillary shoots rapidly in high frequency also. Thus the novel procedure of microrhizome induction in *K. galanga* using AgNPs provides the potential of rapid *in vitro* propagation of plantlets for commercial plantations.

Anatomical studies

Microrhizome formation was further confirmed by anatomical studies of microrhizomes and mother rhizomes of *K. galanga*. Transverse sections of mother and microrhizomes showed a good amount of oil cells; the colour ranges from light yellow to dark yellow (Fig. 4). Number oil cells present in medulla region and cortex region were calculated separately. The results shows significant difference in number of oil cells in different treatments with AgNPs. Here in T_2 (25 mgl^{-1} AgNPs) a good amount of oil cells were present in both medulla (88.10 ±0.32 oil cells) and cortex (92.04±0.33 oil cells). More or less similar number of oil cells present in mother rhizomes. However the number of oil cells present in other treatments using AgNPs were less when compared to the mother rhizomes. This is in line with the findings of oil cells in *Curcuma longa* [32] and also in *Kaempferia rotunda*, where the number oil cells present in field grown plant were 38.0 in outer zone, 38.6 in inner zone, 21.8 in outer zone of microrhizomes and 20.8 in inner zone of the same [33].

Table 5: Oil cells in the rhizome samples of *K. galangal*

Samples	Number of oil cells	
	Medulla region	Cortex region
Mother rhizomes (Control)	100.12±0.12[a]	104.12±0.23[a]
T_1 (12.5)	70.08±0.30[c]	88.31±0.12[c]
T_2 (25)	88.10±0.32[b]	92.04±0.33[b]
T_3 (50)	25.15±0.20[d]	24.11±0.18[d]
T4 (100)	16.23±0.11[e]	18.25±0.13[e]
T_5 (200)	13.20±0.20[f]	16.30±0.31[f]

*Data represents mean values of ten replicates repeated thrice, recorded after 4 weeks of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$.

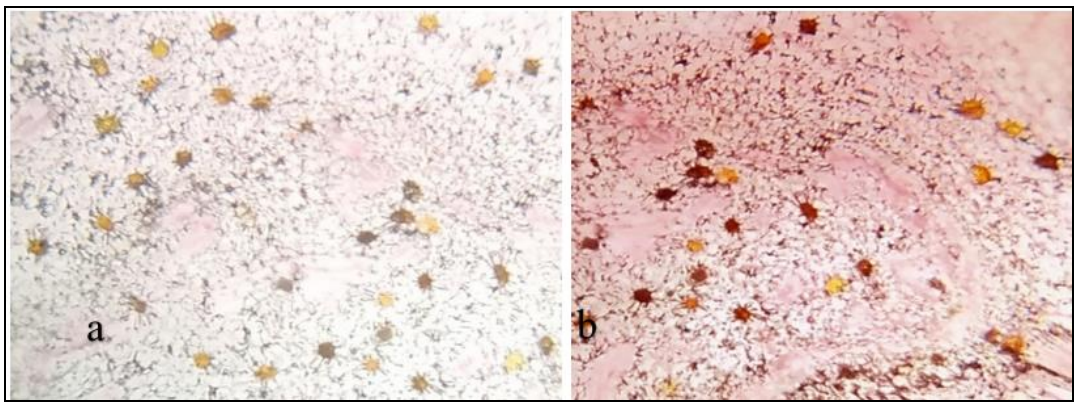

Fig 4: T. S. of rhizome samples showing oil cells in (a) Mother rhizomes, (b) Microrhizomes

Conclusion

The present study reveals that AgNP treatment especially *T2* (25 mgl^{-1} AgNPs) along with 6% (w/v) sucrose concentration and incubation under 8 hr photoperiod for initial 4 weeks and subsequent dark period was found to be ideal for inducing microrhizomes in *K. galanga.* Microrhizome induction occurred in *K. galanga* because of the synergistic effect of AgNP-, high sucrose (6%) and dark period incubation. Anatomical studies confirm the presence of oil cells in microrhizomes. As rhizomes are inferred as the economically useful part in this species, the protocol established here can further be extended for pathogen-free

microrhizome induction and their exploitation for the conservation and sustainable utilization strategies.

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