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# Micropropagation and *In vitro* Flowering of a Biodiesel Plant Niger (*Guizotia Abyssinica* Cass.)

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**ABSTRACT**
*The influence of BAP, Kn and $GA_3$ applied successively on in vitro regeneration and flower bud formation in Guizotia abyssinica Cass. has been investigated. The leaf and internode were isolated from seedlings raised in vitro. High frequency of shoot regeneration and high number of shoots per regenerating explants were obtained on MS medium supplemented with BAP ($1mgl^{-1}$). Proliferated shoots were elongated on same medium and well developed shoots were rooted on half strength MS. In vitro flowering was observed on MS medium supplemented with a combination of BAP ($0.1mgl^{-1}$) + $GA_3$ ($0.1mgl^{-1}$). The plantlets thus formed were successfully hardened sand, soil and farm yard manure (1:1:1) with 85% survival.*
**Keywords:** *Niger, In vitro flowering, Plant growth regulators, Biodiesel, Guizotia abyssinica*

## INTRODUCTION

Niger (*Guizotia abyssinica* Cass.) is one of the important oil seed crop of India and Ethiopia. It is cultivated to a limited extent in Ethiopia, South Africa, East Africa, West Indies, Zimbabwe and India. In India it is mainly cultivated in tribal pockets of M.P., Orissa, Maharashtra, Bihar, Karnataka and Andhra Pradesh. It is also grown sizeable area in certain region of Arunachal Pradesh, Gujarat, U.P., Tamil Nadu and Rajasthan [1]. Its oil is used to prepare various types of foods, paints and soaps, and as an illuminant. In Europe, the seed is used in the preparation of animal and bird feed. Phytochemicals are biologically active compounds present in natural food including fruits, vegetables, grains, nuts, and seeds that have the potential to prevent or delay the onset of chronic diseases [2]. Niger seed contain phytochemical compounds such as fixed oil, fats, protein, amino acids, and flavonoids [3,4].The oil of the seeds is also used in treating rheumatism. Traditionally, niger sprouts mixed with garlic is used to cure cough. Its oil is also used in birth control and, cooked with spices in the treatment of syphilis [5].

Of late the plant has gained importance as a biodiesel plant. The seeds of the plant are edible containing 27-50% of yellow coloured oil having mild odour bearing pleasant nut like taste [6]. The predominant fatty acid in niger seed is linoleic acid (LA) present in rich amounts (54-85%) [7] & is known to prevent cardiovascular disorder and is, therefore, highly valuable. Other fatty acids are palmitic and stearic acids (7–8%), oleic acid (5–25%) [8]. Despite being nutritionally rich and economical important it has by and large remained a neglected crop [9]. Besides, the plant suffers from low yield due to self incompatibility, lodging, shattering, indeterminate growth habit and instability at higher temperature.

Micropropagation of plants is a well known strategy for efficient production and propagation of elite plant material. It helps in the development and rapid propagation of selected plants with desirable characters in shortest possible time and new cultivars can also be developed by protoplast fusion and genetic modifications [10]. Used in conjunction with classical breeding methods, an efficient *in vitro* shoot proliferation and regeneration system could accelerate cultivar development programs [11]. This can be used to develop transgenic plants following genetic transformation of plant cells and to identify and/or induce somaclonal variants [12]

Flowering is an important phase of plant reproduction where the vegetative meristem is converted into a flowering meristem due to physiological, physical and chemical stimuli. Often flowering occur in tissue culture under *in vitro* condition is known as "*in vitro* flowering" [13].Under natural conditions the transition from the vegetative to the reproductive stage (flowering) in plant occur after maturity [14]. Deliberate flowering in culture can serve as a tool for studying flower induction and development as well as to control breeding programs involving species with a long juvenile period [15].

*In vitro* flowering serves as an important tool in studying flower induction, initiation and the floral developmental process by utilizing plant growth regulators such as cytokinins, gibberellins and auxins [16]. *In vitro* flowering can also reduce the influence of environmental factors and can clarify the key influences affecting the flowering process by controlling environmental factors and the application of plant growth regulators [17].

A number of protocols for plant regeneration and *in vitro* flowering through tissue culture have been developed by different researchers at different times in several plant species. For instance, in *Guizotia abyssinica* [18], in *Ceropegia bulbosa* [19], in *Anthemis xylopada* [20], in *Heliotropium indicum* [21], in *Scoparia dulcis* [22], in *Arachis Paraguariensis* [23], and in *Bacopa chamaedryoides* [24].

The present work was undertaken to study the effect of plant growth regulators such as BAP (6-Benzyl amino purine), Kn (Kinetin) and $GA_3$ (Gibberellic acid) for clonal multiplication and *in vitro* flowering of *G. abyssinica* through leaf and internode explants.

## MATERIALS AND METHODS

Seeds of niger var. JNC 6 were obtained from Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV) Jabalpur. Seeds were soaked in water for 24 hrs and washed by wetting agent labolene (1%) and then rinsed in running water (1 hr). They were then surface sterilized using (w/v) 0.1 % $HgCl_2$ for 6-8 min. followed by three rinses with sterile distilled water. Seeds were then inoculated on basal MS [25] medium.

The explants leaf (1cm) and internode (0.8-1cm) were dissected from 15-20 days old *in vitro* seedling (8 cm). Explants were inoculated under aseptic conditions on Murashige and Skoog (MS) medium containing 3% sucrose, 0.8% agar and supplemented with different plant growth regulators viz. BAP (0.1-5mg/$l^{-1}$), Kn (0.1-5mg/$l^{-1}$) and $GA_3$ (0.1-5mg/$l^{-1}$) individually and in combination. The pH of the media was adjusted between 5.6-5.8 with 1 N NaOH or HCl. The media was dispensed in 18 X 150 mm test tubes (Borosil, India) containing 15 ml of MS medium and plugged with cotton plugs (double-layered muslin cloth stuffed with non-absorbent cotton) before autoclaving at 121 ºC pressure for 15 mins. Before inoculation, the autoclaved medium was left at 25° C for 24 h to check for any visible microbial contamination.

For root induction the *in vitro* regenerated shoots (4-6 cm long) were transferred to ½ strength MS liquid (devoid of agar) containing 3% (w/v) sucrose and Whatman filter paper bridges were used as support. Cultures were maintained in culture racks at 25±1 ºC at a 16hr photoperiod provided by cool white fluorescent tubes (Philips, India).

**Hardening and Acclimatization of plantlets**

*In vitro* regenerated plantlets (3-4 months) possessing well developed shoot and root system were washed with tap water to remove adhering agar without damaging the delicate root system and treated with 1% (w/v) fungicidal (Bavistin) solution for 5 min. The plantlets were then transferred to plastic pots containing sterilized sand, soil and farm yard manure (1:1:1). To maintain humidity, hardened plantlets were completely covered with plastic bags and irrigated regularly with distilled water. After 2 weeks the plastic covers were perforated with small holes. The bags were removed intermittently (5-10 mins) to aid adaptation of *in vitro* plantlets to normal environment conditions. Subsequently exposure time was increased in the following weeks and after 7-8 weeks plastic covers were removed completely. After 8 weeks of hardening the plantlets were transferred to the field.

**Histological studies**

*In vitro* behaviour of explants was evaluated through visual observations and histological studies of the cultured tissue. The tissues were fixed in Formalin-Aceto-alcohols (FAA) solution (90 ml of 70% ethyl alcohol, 5 ml of glacial acetic acid and 5 ml of formalin) [26, 27] for 18 hrs, washed for 30 min with tap water, dehydrated by transferring through an ethanol–xylol series and then embedded in paraffin. Tissues were sectioned at 10µm thickness with microtome, mounted on glass slides, and stained with 0.5% safranin. The slides were dehydrated with successive grades of ethanol and counter stained with 0.5% fast green [28](fast green dissolved in 1:1 clove oil, and absolute

ethanol (v/v). These slides were further differentiated in absolute ethanol and xylol series and cleared with pure xylol and finally mounted with Canada balsam. Histological evaluation of the Section materials were made using Olympus CH20i light microscope and photographed.

**Experimental design, data collection and analysis**

The experiments were set according to completely randomized design and all experiments were performed in triplicates with 12 treatments. The data were recorded within 25-30 days of inoculation. The data were analyzed using analysis of variance (ANOVA) and means were separated at $p$=0.05 level of significance using Duncan's multiple range test (DMRT) using the statistical software IBM SPSS 20.

## RESULTS AND DISCUSSION

Both leaf and internode explants of *G. abyssinica* responded within a week of culturing with basal swelling. The leaf explants became swollen along the margins of their cut surfaces and two weeks later developed green shoots without callus formation. The formation of shoot buds occurred on the abaxial surface, when in contact with the medium. Internode explants swelled and turned brown in colour and after 3-4 weeks produced green shoots with little callus formation.

Shoot regeneration occurred in response to all growth regulator combinations tested. A rhizogenic response was initiated from leaf and internode explants on basal MS medium. Shoot induction was dependent upon the concentration of cytokinin supplemented in the medium. Cytokinin probably worked as a signalling molecule to activate totipotent cells of callus for shoot organogenesis resulting in multiple shooting responses. The regeneration parameter viz. percentage of shoot initiataion, number of shoots and average shoot length were controlled by the type and concentration of the growth regulator employed. Direct multiple shoots were induced on MS medium supplemented with different hormonal concentration/combination. Maximum frequency of shoot induction and multiplication from both explants was found to be more pronounced when MS media was supplemented with BAP ($1mgl^{-1}$) (Table 1, Fig.8-9 & 12-13). However, the percentage of bud break and induction declined with the increase in BAP concentration. Kinetin supplemented media on the other hand were less responsive The superiority of BAP over other cytokinins for shoot bud regeneration in the present study is corroborated with earlier published reports [9, 29]. Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue [30]. The medium supplemented with Kn ($5mgl^{-1}$) showed morphogenetic response only from leaf explants.

**Table 1. Effect of BAP, KN and $GA_3$ (alone) and in combination on shoot proliferation in Leaf and Internode explants of *Guizotia abyssinica* Cass.**

| PGRs | Conc. ($mg/T^{-1}$) | FSI | | MSN | | MSL | |
|---|---|---|---|---|---|---|---|
| **BAP** | | **Leaf** | **Internode** | **Leaf** | **Internode** | **Leaf** | **Internode** |
| | 0.1 | 27.77±2.26$^{b}$ | - | 1.38±0.3$^{b}$ | - | 0.41±0.3$^{ab}$ | - |
| | 0.5 | 55.55±2.26$^{c}$ | - | 3.24±0.3$^{c}$ | - | 0.63±0.3$^{ab}$ | - |
| | 1 | **88.88±0.01$^{d}$** | **69.44±0.01$^{b}$** | **5.60±0.6$^{d}$** | **2.13±0.3$^{b}$** | **1.10±0.4$^{b}$** | **0.91±0.4$^{b}$** |
| | 5 | - | - | - | - | - | - |
| **Kinetin** | 0.1 | - | - | - | - | - | - |
| | 0.5 | - | - | - | - | - | - |
| | 1 | - | - | - | - | - | - |
| | 5 | 77.77±2.26$^{b}$ | - | 2.24±0.1$^{b}$ | - | 0.30±0.01$^{b}$ | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **BAP+$GA_3$** | 0.1+0.1 | $30.55\pm2.26^{cd}$ | - | $1.05\pm0.2^{bcd}$ | - | $0.12\pm0.01^{b}$ | - |
| | 0.1+0.5 | $61.10\pm4.53^{f}$ | - | $1.99\pm0.1^{e}$ | - | $0.20\pm0.01^{c}$ | - |
| | 0.1+1 | $13.44\pm2.09^{abc}$ | - | $1\pm0.1^{bc}$ | - | $0.07\pm0.01^{bc}$ | - |
| | 0.1+5 | $3.05\pm2.16^{ab}$ | - | $1.13\pm0.01^{d}$ | - | $0.09\pm0.01^{b}$ | - |
| | 0.5+0.1 | $38.88\pm2.26^{de}$ | - | $2.80\pm0.2^{f}$ | - | $0.35\pm0.01^{d}$ | - |
| | 0.5+0.5 | $55.55\pm4.53^{f}$ | - | $2.85\pm0.2^{f}$ | - | $0.70\pm0.01^{e}$ | - |
| | 0.5+1 | **$77.77\pm2.26^{g}$** | **-** | **$3.97\pm0.2^{h}$** | - | $0.88\pm0.01^{f}$ | - |
| | 0.5+5 | $13.44\pm2.09^{abc}$ | - | $1.13\pm0.1^{cd}$ | - | $0.34\pm0.01^{d}$ | - |
| | 1+0.1 | $22.0\pm2.45^{bcd}$ | - | $0.94\pm0.1^{b}$ | - | $0.21\pm0.02^{c}$ | - |
| | 1+0.5 | $52.44\pm2.27^{ef}$ | - | $3.10\pm0.2^{g}$ | - | $0.25\pm0.01^{d}$ | - |
| | 1+1 | $25\pm2.27^{cd}$ | - | $1.02\pm0.1^{bcd}$ | - | $0.08\pm0.01^{bc}$ | - |
| | 1+5 | $22.0\pm2.45^{bcd}$ | - | $0.58\pm0.1^{a}$ | - | $0.12\pm0.01^{b}$ | - |
| **Kn+GA3** | 0.1+0.1 | - | - | - | - | - | - |
| | 0.1+0.5 | - | - | - | - | - | - |
| | 0.1+1 | $38.88\pm2.26^{e}$ | - | $0.99\pm0.2^{b}$ | - | $0.21\pm0.01^{c}$ | - |
| | 0.1+5 | $47.22\pm2.27^{f}$ | - | $1.47\pm0.1^{c}$ | - | $0.25\pm0.01^{d}$ | - |

**Experiment was repeated thrice with 12 replicates. Values represent mean ± standard error. Mean values followed by different superscript letters is significantly diffrenet at P≤0.05,as detrmined by Duncan's Multiple renge Test.**

Singular supplements of $GA_3$ regardless of concentrations were unsuitable because they yielded inferior shoot regeneration response compared to that with BAP alone. However, $GA_3$ at an optimal concentration of $1mgl^{-1}$ when added in conjuction with BAP ($0.5mgl^{-1}$), markedly enhanced the frequency of bud break (77.77%) in addition to inducing a quick bud break (7-8 days) (Table 1). Similar results were also obtained in *Ocimum basilium* [31] and in *Tylophora indica* [32].

Flower bud initiation started on the apex and axil of some branches of *in vitro* regenerated shoots (Fig. 14-16) and maximum frequency of flowering (65%) was obtained on BAP ($0.1mg/l^{-1}$)+ $GA_3$ ($0.1mg/l^{-1}$) after 3 weeks in this plant. An average of 8-10 flower buds were formed per explants on this concentration (Fig. 14-15). This result differ from the earlier report [18] which showed a lower frequency (40%) of *in vitro* flowering. Higher concentrations of $GA_3$ ($>1mgl^{-1}$) were no longer found stimulatory when combined with BAP for bud break although lower ones stimulated this process. Similar results were also obtained in *Torenia* [33] The effect of BAP and $GA_3$ on flowering depends to a considerable degree on the concentration applied. Cytokinin and gibberellin included in a medium in proper succession played an essential role in the mechanism of flowering in *G. abyssinica*. It seems possible that the influence of phytohormones on flowering may be related to the change in growth correlations within meristem of an apical shoot. The role of cytokinin is to initiate mitosis and that of gibberellins is to affect cell elongation and growth. Under *in vitro* conditions, the supply and balance of the type of cytokinin and gibberellin can favor floral development and maturation. The combination of cytokinin and gibberellin supported *in vitro* flowering in *Rauvolfia tetraphylla* [34], *Pharbitis nil* [35], *Panax ginseng* [36-37].

Structural analysis is an important step in the study of organization and changes in the plant body, and it is an extremely useful approach in the study of plant morphogenesis [38] Therefore histological studies were conducted on responding leaf and internode segments to trace the origin of multiple shoot buds. The floral morphology of the *in vitro* plants was normal and identical to the donor plants. Gradual processes of flower bud initiation to complete flower formation was also studied histologically (Fig 19c & 19d). Longitudinal sections of explants showed the development of meristematic structures in the form of a dome shaped apex with leaf primordia (Fig 19(a)). These cells further

differentiated and formed multiple shoots (Fig 19(b)). The different stages observed during floral morphogenesis *in vitro* were evidenced by similar stages in mother plant.

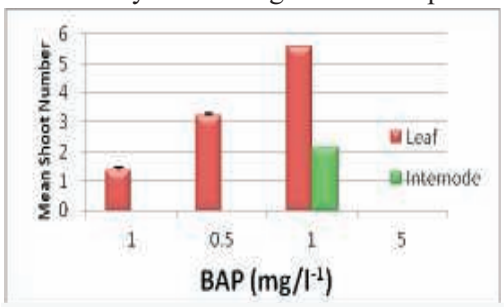



**Fig. 1 Effect of BAP on Mean Shoot number in Leaf & Internode explants of *G. abyssinica* Cass. *in vitro***

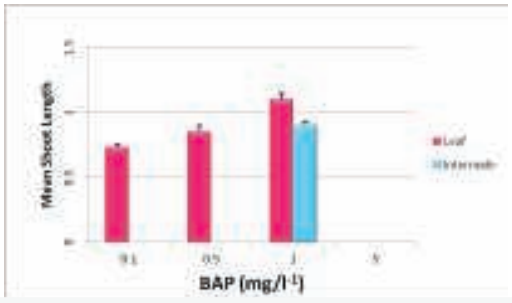



**Fig. 2 Effect of BAP on Mean Shoot length in Leaf & Internode explants of *G. abyssinica* Cass. *in vitro***

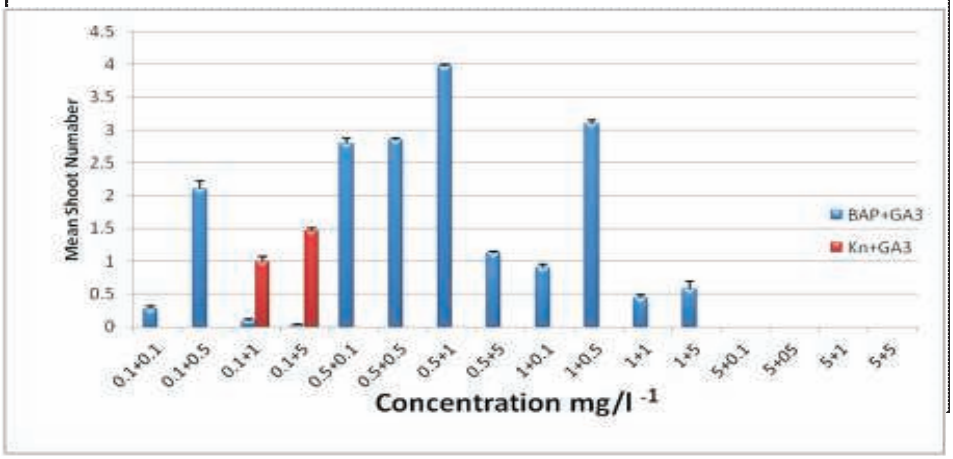



**Fig 3 : Effect of BAP +GA $_3$ and Kn + $GA_3$ on Mean Shoot number in Leaf explants of *G. abyssinica* Cass. *in vitro***

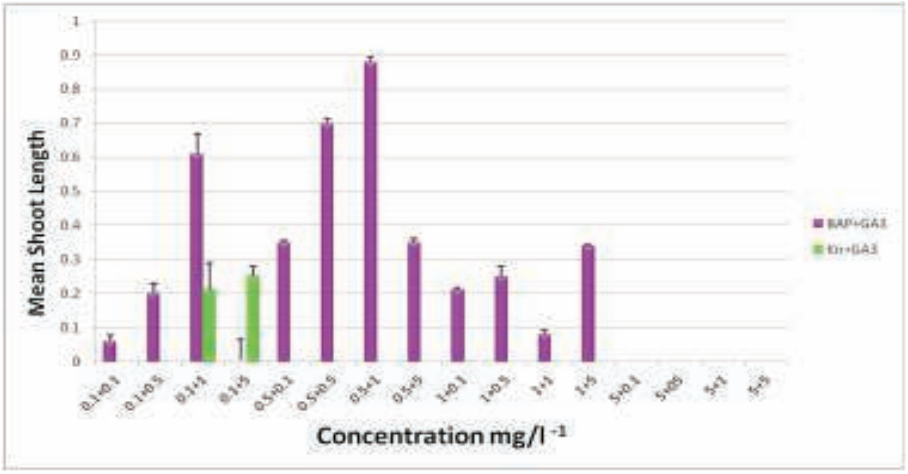
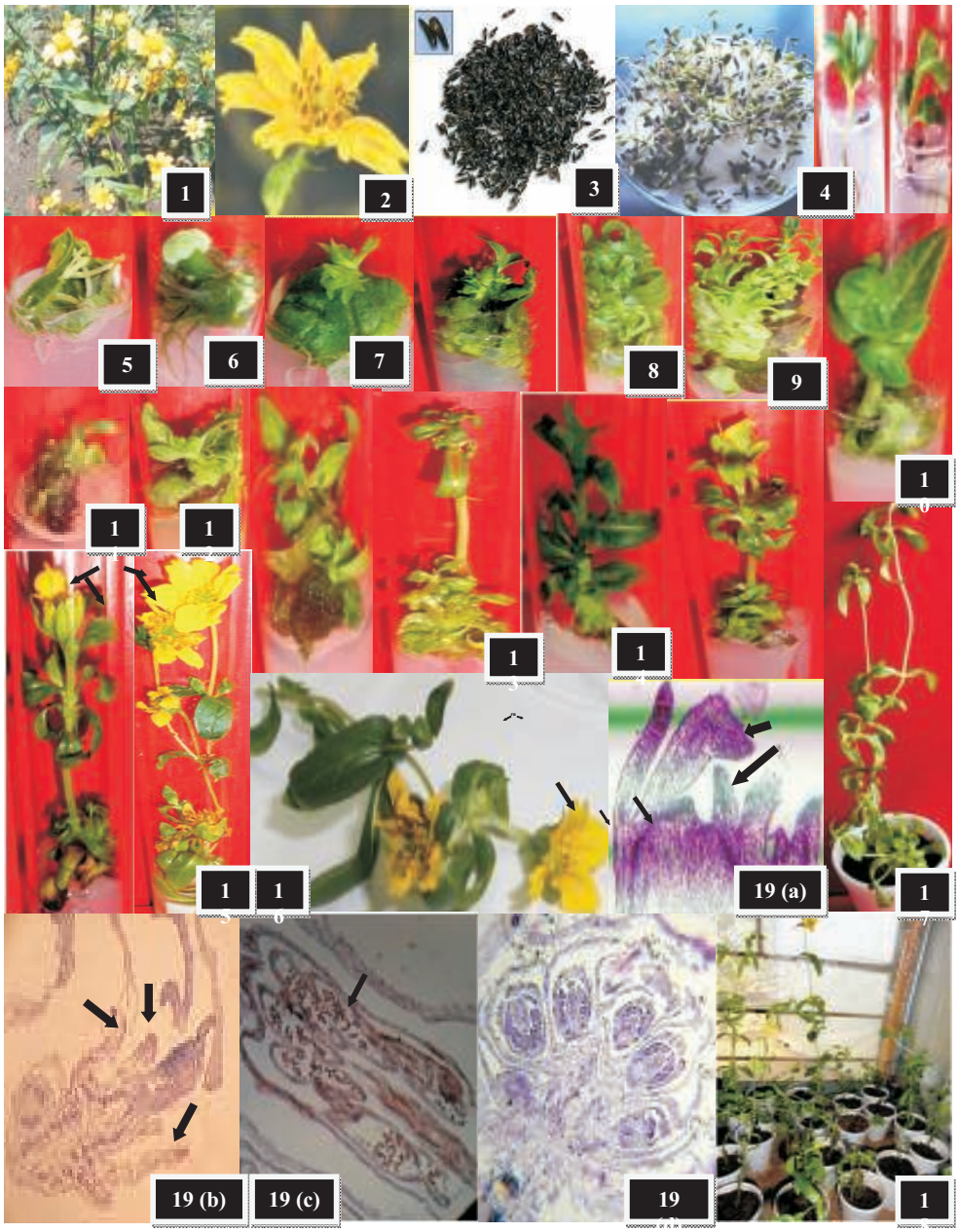



**Fig 4 : Effect of BAP +GA $_3$ and Kn + $GA_3$ on Mean Shoot length in Leaf explants of *G. abyssinica* Cass. *in vitro***




**Description-** ***In vitro*** **Regeneration and flowering in** ***Guizotia abyssinica***

Fig 1-3 Natural Plant, Flower and Seed of *Guizotia abyssinica.*
4. Seed germination on Filter paper and Basal medium.
5-6. Regeneration on basal medium from leaf and internode explants.
7. Shoot initiation with BAP ($1mgl^{-1}$) 2 wks from leaf explants.
8. Multiple shoot formation with BAP ($1mgl^{-1}$) 3 wks from leaf explants.
9. Multiple shoot formation with BAP + $GA_3$ ($0.5+1mgl^{-1}$) 3 wks from leaf explants.
10. Shoot elongation with BAP ($1mgl^{-1}$) 4 wks from leaf explants.
11. Shoot initiation with BAP ($1mgl^{-1}$) 2 wks from internode explants.
12. Multiple shoot formation with BAP ($1mgl^{-1}$) 3 wks from internode explants.
13. Shoot elongation with BAP ($1mgl^{-1}$) 4 wks from internode explants.
14- 16. Flowering of *in vitro* regenerated plantlets.
17-18. Hardened *in vitro* regenerated plants in poly cup.
19 (a). Histological section showing shoot initiation and development.
19 (b). Histological section showing multiple shoot formation.
19 (c). Histological section showing Pollen development.
19 (d). Histological section showing complete flower development.

SAM- Shoot apical meristem
LP- Leaf Primordium
MS- Multiple shoots
IF- *In vitro* flower
FB- Flower bud
P- Pollen grain

The *in vitro* regenerated shoots were transferred to ½ MS medium without auxin. Root formation from the basal utportion of the shoots were observed after one week and rooting frequency gradually increased overtime and reached maximum after 4 week of culture. The success of *in vitro* regeneration relies on the rooting percentage and survival of the plantlets in the field conditions. The incidence of root formation on auxin free medium may be due to the availability of endogenous auxin in *in vitro* shoots [39]. Similar results were obtained in *Dendrobium microbulbon* [40], *Stevia rebaudiana* [41] and *Bacopa chamaedryoides* [24]. Well developed plantlets were transferred to poly cups containing sterile sand, soil and farm yard manure (1:1:1) under high humidity. Approximately 85% of hardened plantlets survived. After 4 weeks hardened plantlets were transferred to field. There was no detectable variation among the potted plants in terms of morphological and growth characteristics.

## CONCLUSION-

The protocol presented here demonstrates an efficient plant regeneration protocol from leaf and internode explants which has important implications for the genetic transformation of this multipurpose plant. Moreover, *in vitro* flowering obtained in this plant, makes this protocol highly efficient for an oil yielding crop.BAP in combination with $GA_3$ played an important role in the transition from a vegetative to a floral state but there is certainly other biochemical and environmental factors involved in this process that needs to be investigated further.

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