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Localization of Saponins Accumulation in *Yucca Gloriosa* Variegata in Intact Tissues and Their Tissue Cultures by Using Fluorescence Microscopy

Ansam, G. Abdulhalem[1,*], Bushra Alwash[2] and Kadhim M. Ibrahim[3]

[1]Department of Biology, College of Science, University of Baghdad, Iraq
[2]Department of Biology, College of Science for Women, University of Baghdad, Iraq
[3]Department of Biotechnology, College of Science, Al-Nahrain University, Iraq
*Email: Ansam.ghazi@sc.uobaghdad.edu.iq

Abstract

Steriodal saponins are important active components of *Yucca gloriosa* Variegata, a medicinally important plant. The detection and localization of saponins accumulation is very necessary to determine which plant organ or tissue accumulates high levels of such important compounds. In this study, it has been reported for the first time the accumulation of saponins in leaves, rhizomes and shoot apex of *Y. gloriosa* Variegata and their *in vitro* cultured tissues (calli, direct and indirect regenerated shoots and rhizomes/roots) through histological sections using fluorescence microscope. This method presents fast and significant manner for saponins accumulation sites due to its high sensitivity and the selectivity of analyzing signals. Additionally, the intensity of fluorescence distinguishes the level of saponins in these tissues. Specimens were fixed, embedded in paraffin, and stained with aniline blue (390 nm excitation and 480 nm emission) then viewed with BX53 (Olympus)fluorescence microscope. Photographs were taken using a high sensitivity digital camera. Results showed high level of saponins accumulation appeared particularly in indirect regenerated leaf (initiated from callus cultures) and in callus treated with Thidiazuron (TDZ). Saponins content was variable during different periods of callus growth.

Keywords: *Yucca gloriosa*, saponins, fluorescence microscopy, tissue culture, histology

1. Introduction

Yucca gloriosa Variegata of family Agavaceae has many common names including variegated Spanish dagger, Mound-lily or Palm lily. It is familiar as ornamental and decorative plant characterized as an extremely hardy and drought resistant. Apart from being a source of wide range of useful products, this genus has found reputed place in folk medicine. It is used for ulcer treatment, jaundice, asthma, bronchitis, soothe joint pain, bleeding due to its ant platelet effect and has heapto-protective activity, antioxidant activity, and used as anti-helminthic and antimicrobial (antifungal, antiviral and antibacterial) agent. Additionally, it has been used as a natural foaming agent. Some important steroidal saponins, which have been isolated from leaves and rhizomes, are used as purgative and detergent [1, 2, 3, 4, 5, 6]. The interest of this plant is increasing because all parts of *Y. gloriosa* are rich in steroidal

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compounds [7, 8, 9, and 10]that have wide applications in pharmaceutical industry mainly as anticancer drugs [11, 12]. Steroidal glycosides including new organic compounds have been isolated from plant leaves, flowers, and rhizomes [13].*Y. gloriosa*is also used as a source of the sapogenintigogenin which is used as raw material for the synthesis of steroidal hormones[14]. In addition, tigogenin has anti-arthritic activity, anti-obesity effect by inhibiting adipocyte formation [15] and has anti-diabetic action [16].

Saponins are glycosylated natural products, a vast group of structurally diverse glycosides of the plant kingdom and of some marine organisms and insects [17]. They have a high molecular weight and able to interact with cell membranes and are able to decrease the surface tension of an aqueous solution [18], and these surface active properties distinguished them from other glycosides, beneficiary from them in industrial applications as foaming and surface active agents. They contain a steroidal or triterpenoidaglycone and one or more sugar chains.

The presence of saponins (Foaming glycosides) are reported in more than 100 plant families, in both wild and cultivated plants. Triterpenoid saponins are occur mainly in dicotyledonous plants but also in some monocots, whereas steroid saponins found mainly in monocots, such as the Agavaceae, Lilliaceae, Asparagaceae, Droscoraceae, Trilliaceae and Smilacaceae and in certain dicotyledons, such as Fabaceae and Solanaceae[19, 20].While, the main dietary sources oftriterpenoidsaponins have been detected in many legumes such as soybeans, beans, peas, lucerne, etc. and also in alliums, tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponinsare found in oat, capsicum pepper, eggplant, tomato seeds, asparagus, yam, fenugreek, *Yucca* and ginseng.There are many factors may effect on the accumulation of saponins in plants such as the cultivar, physiological state, the age, and geographic location [21].However most studies on *Y. gloriosa*have mainly focused on the components analysis, pharmacology and medicinal treatments, in the present study we first used the histological method by using fluorescence microscopy to investigate steroidal saponins in plant organs and its tissue cultures.

Despite there are only few studies on *in vitro* culture of *Yucca* most of them achieved in order to micropropagatethese species, *in vitro* cultures of *Y. gloriosa*could form alternative method for plant proliferation as well as for saponins production.The aim of this study was toestablish plant tissue cultures of *Y. gloriosa* Variegate protocol using growth regulators then determine the optimal plant part that accumulates steroidal saponinsafter investigatingsaponin production sites intercellular of intact plant and tissue cultures by using fluorescent microscope.

2. Materials and methods

2.1. Plant material and culture conditions

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Buds (terminal and auxiliary) segments were taken from plants growing in a greenhouse. They were collected and washed with tap water for 30 min and then transferred to air flow cabinet, surface sterilized with 70% ethanol for 30 sec, submerged in 4% Clorox for 15 min and then rinsed three times with sterile distilled water. Explants were aseptically, cultured on MS medium with different combinations of plant growth regulators, incubated in a growth chamber at 25 ± 2°C in dark for 1 week, and then transferred to light conditions under 16 hrs illumination (2000 Lux, daylight fluorescent tubes). Agar was added after adjusting the pH to 5.8. To determine the best auxin and cytokinin combination, the explants were inoculated into MS (Murashieg and Skoog) medium fortified with different concentrations of 6-Benzyladenine (BA), α-Naphthaleneacetic acid (NAA) and TDZ in two separate experiments. Different concentrations of each plant growth regulator were combined together and they were examined as the following:

2.2. Callus induction from bud culture (terminal and auxiliary buds)

Shoot tips/auxiliary buds were excised (3-5 mm) from aseptic plants and cultured on MS medium supplemented with factorial combinations of 0.1, 0.2, 0.3 or 0.4 mg/L BA plus 1.0, 1.5, 2.0 or 2.5 mg/L NAA. After six weeks, callus fresh weight was recorded.The medium was supplemented with another type and concentrations (0.0, 0.1, 0.5, 1.0 mg/L) of the cytokinin TDZ added to the combination of BA 0.1 mg/L + NAA 1.5 mg/L as a control medium for callus induction from terminal and auxiliary buds by using different types of cytokinins. Ten replicates were used per every type of callus induction medium. A completely randomized design was used, means were compared and least significant differences were calculated at 0.05 of probability using statistical analysis system SAS [22].

2.3. Plant regeneration

Pieces weighting 0.5 g of calliwere dividedand inoculated separately in tubes containing fresh MS medium supplemented with combinations of 9 mg/LBA and 0.1 mg/LNAA to initiate shoots and routinely subcultured every 6 weeks.Tubeswere kept in a growth chamber under the same conditions mentioned above. After 12 weeks, regenerated plantlets 2-4 cm in length were separated and transferred into a rooting medium containing 1.0 mg/L of Indole 3-butyric acid (IBA) and half power of MS medium macronutrients. Rootingwasobtainedafter 8 weeks.

2.4. Histological studies

Fresh leaves, rhizomes, shoot apices of *Y. gloriosa*Variegata and its tissue cultures (calli, regenerated shoots, rhizomes)were fixed in formalin-acetic acid-alcohol (FAA), embedded in paraffin, cut into 10 μm sections on a rotary microtome and stained with aniline blue 0.1% in absolute n-butanol and buffered at pH 8.2, fluorochrome, which was prepared immediately 30 minutes before use, rinsed with phosphate buffered saline (PBS), and then post-stained with toluidine blue (0.5% in

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PBS 1X at 7 pH) for 2 minutes to minimize unspecific staining by aniline blue and diminish the intensity of cell wall autofluorescence. Sections were rinsed with PBS again to remove the excessive stain. Then, the slides were submerged in xylene for 10 minutes. Mounting medium was consisted of 90% glycerol- PBS mixture at pH 8-9. This was added to glass slides in order to prevent drying of samples and influence the quality of imaging. Sections were monitored under fluorescence microscope BX53 (Olympus). Ultraviolet illumination was utilized. After excitation with light at 390 nm wavelengths, emitted fluorescence was photographed using a filter for the detection of fluorescence light at 480 nm wavelengths by a high sensitivity digital camera.All specimens were investigated under 40X objective lens.

3. Results and discussion

3.1. Callus induction

Various combinations of BA and NAA were added to MS medium to determine the most suitable combination of plant growth regulators for callus induction from buds. During the 6 weeks culture period, the bud explants formed callus at some concentrations of BA and NAA, while no responses after increasing BA and NAA concentrations in the medium (Table 1). The combination of 0.1 mg/L BA and 1.5 mg/L NAA recorded 1287.9 mg of callus fresh weight. The response of explants for callus induction was superior at the concentrations 0.2 mg/L BA and 1.5 mg/L NAA and this combination produced 2649.8 mg which was significantly the highest fresh weight in this culture. The presence of both NAA and BA in the medium is necessary for optimum callus formation. This result was observed previously in*in vitro* cultures for other plant species by Arivalagan*et al*. [23] who assured that high concentrations of auxin and low concentrations of cytokinin added to MS medium stimulated callus formation [23].

Table 1: Effects of BA and NAA combinations on callus fresh weights (mg) induced from bud explants after six weeks of culture.

BA (mg/L)	NAA (mg/L)				Mean
	1.0	1.5	2.0	2.5	
0.1	0.0	1287.9	996.3	0.0	571.05
0.2	653.1	2649.8	1023.7	742.9	1267.37
0.3	0.0	0.0	0.0	0.0	0.0
0.4	0.0	0.0	0.0	0.0	0.0
Mean	163.28	984.43	505.00	185.73	---
LSD (P≤0.05)	NAA= 102.46; BA= 102.46; NAA×BA= 163.94				

To produce more masses of callus, Thidiazuron (TDZ)was used at different concentrations. Callus induction from buds of *Yucca gloriosa* demonstrated

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significant differences in masses of callus, depending on the type and concentration of the cytokinin. Faster callus growth and more callus production were obtained on MS medium supplemented with TDZ. It produced a highest fresh weight of callus (reached 4107 mg) at 1.0 mg/L TDZ compared with those cultured on the control medium containing NAA 1.5 mg/L + BA 0.1 mg/L while TDZ at low concentrations (0.1 and 0.5 mg/L) produced direct shoots (see Table 2; Fig. 1C, D). Similar results were obtained by Atta-Alla and Van Staden [24] who reported that treatment with TDZ produced excessive callus in *Y. aloifolia* [24] and Tawfix and Mohammed [25] who recorded a high percentage of explants developing callus on a medium containing 4.5 µM TDZ [25]. This may due to the role of TDZ in enhancing DNA synthesis and thereafter RNA resulting in protein synthesis and ultimately more cell division. Other reports explained that TDZ has similar mechanism to both auxin and cytokinin although, chemically, it is very different from commonly used auxins and cytokinins.

Table 2: Effect of TDZ added to MS medium supplemented with a combination of BA 1.0 mg/L and NAA 1.5 mg/L on callus fresh weight initiated on bud explants after three weeks of culture.

BA (mg/L)	NAA (mg/L)	TDZ (mg/L)	Callus fresh weight (mg)
0.1	1.5	0.0	1357
0.1	1.5	0.1	-
0.1	1.5	0.5	-
0.1	1.5	1.0	4107
Means			2660
LSD (P≤0.05)			323.8

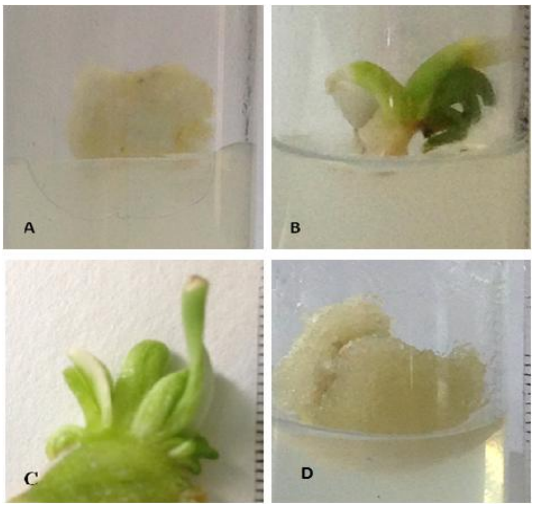

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Fig. 1: Callus induction on *Y. gloriosa* bud explants. A: Treatment of bud explants with 0.1 mg/L of BA and 1.5 mg/L NAA without TDZ; B: Treatment of bud explants with 0.1 mg/L of TDZ, 0.1 mg/L of BA and 1.5 mg/L NAA; C: Treatment of bud explants with 0.5 mg/L of TDZ, 0.1 mg/L of BA and 1.5 mg/L NAA ; D: Treatment of bud explants with 1.0 mg/L of TDZ, 0.1 mg/L of BA and 1.5 mg/L NAA.

A number of biological (physiological and biochemical) events in cells are induced or enhanced by TDZ. However, a variety of reports showed how morphogenic events are induced by application of TDZ. Other reports showed that TDZ may modify plant endogenous growth regulators, either directly or indirectly (for example, TDZ treatment could result in an increase in endogenous auxin levels which usually stimulate callus formation) and produce reactions in cell/tissue necessary for its division/regeneration [26], modification in cell membrane, energy levels, nutrient absorption, transport and assimilation may be possible [27].It is concluded from the current result that treatments with TDZ produce excessive callus formation of *Y.gloriosa* as has been elucidated before.

3.2. Plant regeneration

High response of shoot proliferation was obtained in the presence of BA (9.0 mg/L) and NAA (0.1 mg/L) in MS medium after 12 weeks of *in vitro* culture. Establishment of shoot proliferation with increasing cytokinin accompanied with low auxin concentrations in the medium was consistent with other studies on many Agavaceae plants [28, 29].In tissue culture, cell fate in the callus is determined by different ratios of exogenous cytokinin and auxin referring to the importance of these ratios and their interaction on organ regeneration [30].Additionally, the endogenous level of growth regulators greatly affects the demand of exogenous growth regulators in the plant system [31] andplays a main role in organ growth and patterning[32].

Shoots proliferated from calli cultures were separated and cultivated on MS medium together with IBA (1.0 mg/l) for rooting. After 8 weeks, regenerated shoots successfully rooted in this medium. The obtained plants showed a very similar morphology to greenhouse-grown plants of the same age. For this reason, the system can constitute an efficient protocol for the micropropagation of *Y.gloriosa*. This is of an interest as *Y. gloriosa* tends to grow slowly and propagation of this plant by cuttings and offsets produces few plants [33].

3.3. Microscopic investigation of steroidal saponins

The accumulation of saponins in leaves, rhizomes and shoot apex of *Y. gloriosa* Variegata and their *in vitro* cultured tissues (Calli, direct and indirect regenerated shoots and rhizomes/roots) were examined through histological fluorescent localizations using fluorescence microscope. The results showed that saponins distribution in intact plant and different developmental stages of culture parts was varied.

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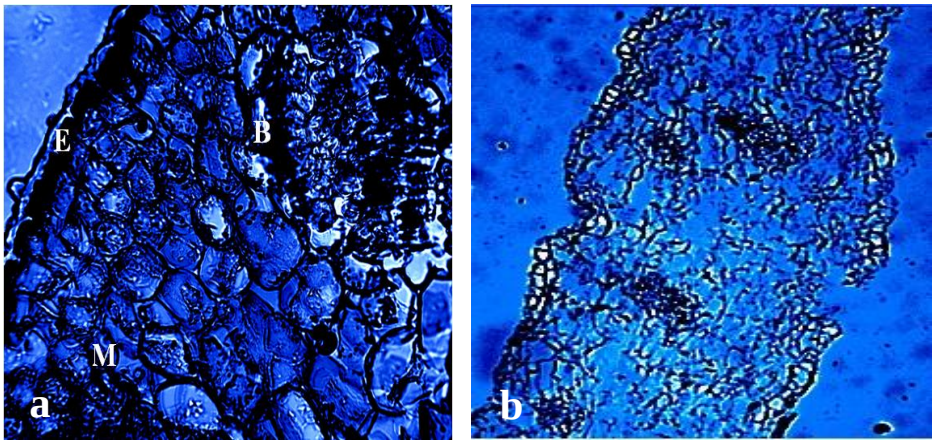
3.3.1. Localization of saponins accumulation in the leaves:

In tender young leaves, obvious florescence was shown in the mesophyll layers and to a smaller extent in epidermal cells in addition to the bundle sheath cells that were appeared high level of florescence (Figure 2-a). Similar pattern of saponins accumulation was noted in tissues of plantlet leaves obtained from indirect regeneration cultures which showed large distribution of the fluorescence through the mesophyll layers and in xylem tissues (Figure 2-c, d). Whereas the location of saponins accumulation was restricted in the epidermal layer with little amounts observed in the mesophyll cells of direct regenerated leaves as viewed in figure (2-b). This may due to the influence of growth regulators and plantlets age. Zhao *et al.* [34] referred that the distribution of triterpenoidsaponinsis varied in different plant organs according to the plant age and the amount of saponins obviously differed among the organs [34, 35].

3.3.2. Localization of saponins accumulation in rhizomes

The cross section of the plant rhizome, the outer layers were free of the fluorescence saponin locations and only accumulated in pith cells (Figure 2-e) while in the rhizomes obtained from *in vitro* cultures, the presence of saponins was distinctly observed in the cortical tissues and to a lesser extent in epidermal cells as displayed in Figure 2-f. Mylona*et al.* [36] revealed that autoflurescentsaponins (avenacin A-1) after staining with aniline blue appeared to be localized in the vacuoles of root epidermal cells in wild and mutant types of Oat with different modes of accumulation in addition to the accumulation of steroidal saponins in leaves [36]. Accumulations of saponinsare attributed to the influence of specific genes in the process of glucosylation.



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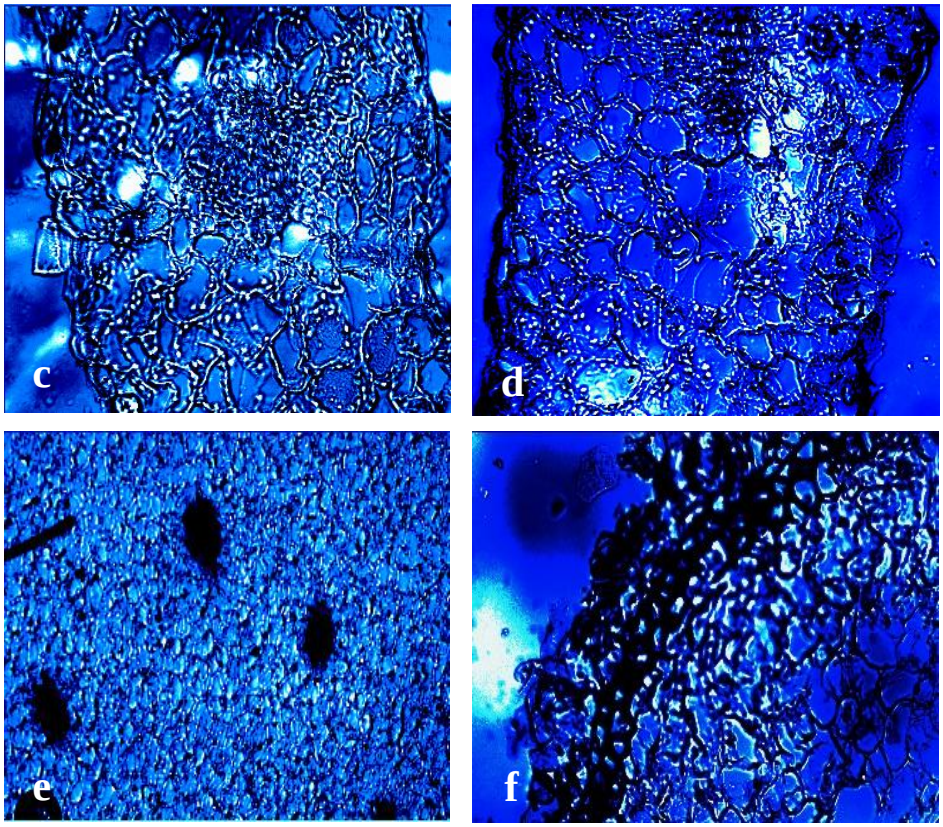

Fig. 2: Florescence micrograph of cross sections of *Y. gloriosa* Variegata vegetative organs in intact plant and its tissue cultures stained with aniline blue flurochrome showing saponins accumulation that gave rise to blue-whitish fluorescence. a: fluorescence of saponins in mesophyll cells and bundle sheath cells of intact plant leaf; b: saponins accumulation in epidermal cells with small amounts in cortical cells of direct regenerated shoot from *in vitro* culture; (c, d) indirect regenerated shoot from *in vitro* culture with distinct fluorescence of saponins accumulation; e: central root region of intact plant; f: saponins accumulation in in cortical cells of root initiated from *in vitro* culture.All specimens were photographed under 40X objective lens.

3.3.3. Localization of saponins accumulation in callus tissues

Callus treatments with different growth regulators were investigated at two different periods. The higher capacity for saponins accumulation was distinguished at the first period in all treatments, whereas the accumulation reduced at the second period as shown in Figure (3: A, B). These results are in agreement with the results of Khanna and Purohit [37] who reported a linear relationship in sapogenin production with the tissue growth through 8 weeks of culture age whereas an obvious decrease happened at 10 weeks [37]. It was also found that the highest saponins production was recorded in callus derived from leaves in *Centellaasiatica* through three weeks then the contents decreased gradually. Possible secretion of saponins into the surrounding medium accompanied with degradation of the products could be the reasons for lowering saponins yield in tissues [38].

Wide distribution of fluorescence locations was manifested in the callus that treated with 0.1 mg/L BA, 1.5 mg/L NAA and 1.0 mg/L TDZ (Figure 3-e, f) but the level of fluorescence decreased in callus tissues that treated with 0.2 mg/L BA, 1.5

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mg/L NAA (Figure 3-c, d) followed by callus tissues that treated with 0.1 mg/L BA and 1.5 mg/L NAA without TDZ (Figure 3-a, b). Optimization of *Yucca glorisa* to tissue culture is crucial for such studies [39].

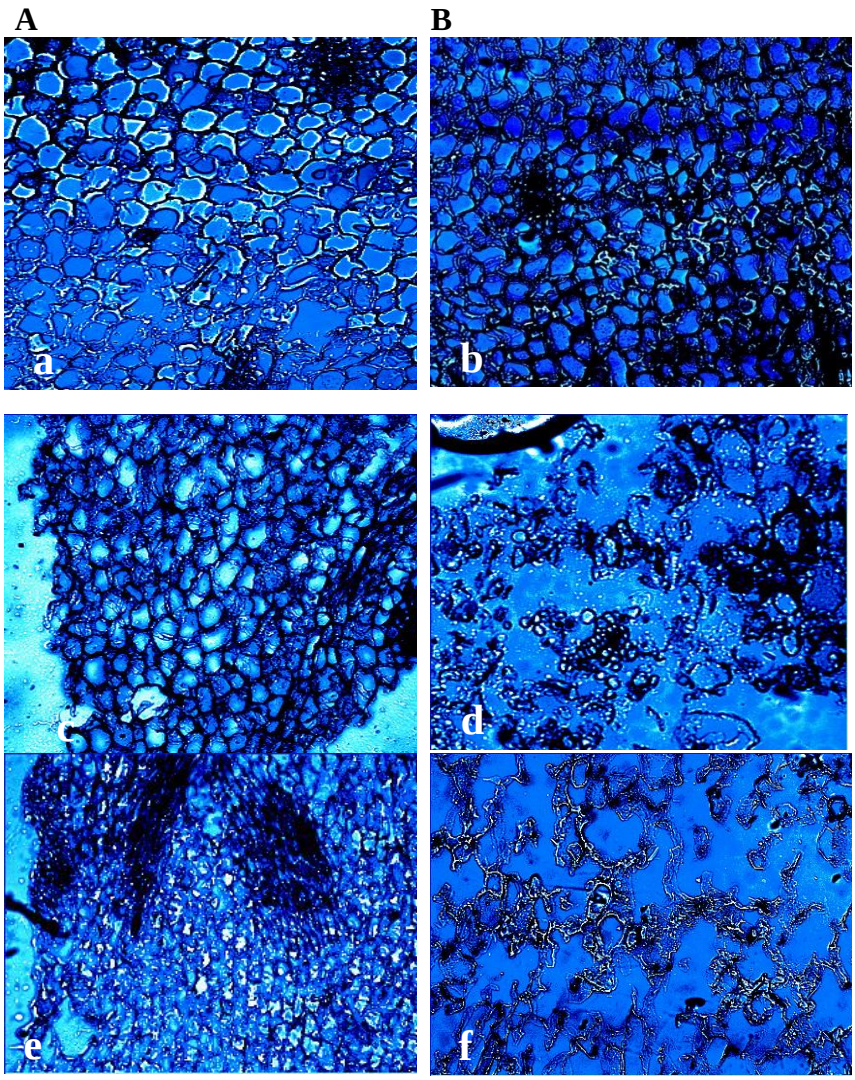

Fig. 3: Florescence micrograph of cross section in *Y. gloriosa*Variegata callus (a, b) treated with 0.1 mg/L BA and 1.5 mg/L NAA; (c, d) treated with 0.2 mg/L BA and 1.5 mg/L NAA; (e, f) with 0.1 mg/L BA, 1.5 mg/L NAA and 1.0 mg/L TDZ showing saponins accumulation giving rise to blue-whitish fluorescence. A: after 3 weeks with the addition of TDZ and 6 weeks without the addition of TDZ; B: after 8 weeks. All specimens were investigated under 40X objective lens.

3. Conclusions

Generally, tissue culture technique is a potential source for plant propagation and for saponins production as secondary metabolites.The use of TDZ at different concentrations in combination with BA and NAA throughout tissue culture can be applied efficiently on large-scale propagation in short time for commercial nurseries through direct regeneration or for increasing the production of secondary metabolites

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via positively callus induction. As the addition of TDZ to the growth medium recorded excessive callus formation, in short time reached three weeks.

Fluorescence microscope presented fast and significant manner to detect and determine the accumulation sites in plant tissues and their distribution within plant organs as well as the intensity of fluorescence distinguishes the level of secondary metabolites in these tissues. Fluorescence micrography showed high level of saponins accumulation in indirect regenerated leaf (initiated from callus cultures). Among the four callus sections investigated, callus that treated with TDZ accumulated the highest amount of saponins. Saponins content is variable during different periods of callus growth.

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