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STUDY FORMATION OF CALLUS FROM *DATURA SP.* AND THE EFFECT OFABIOTIC ELICITORS ON CALLUS MASS

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ABSTRACT

The study aimed to establish tissue culture protocols for *Datura* species. Callus tissues of *Datura stramonium* and *D.innoxia* were initiated on Murashige and Skoog (MS) medium supplemented with various combinations of auxins {2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA)}, and cytokinin {6-benzyl amino purine (BAP), Kinetin} were used. Tissue culture was initiated from leaves and sterile seedlings after breaking seed dormancy. Callus culture was established and the most effective growth regulators were Kin, IAA and 2,4-D for *D.stramonium* leaf explant, The concentration 1mg/l Kin with 0.5mg/l 2,4-D was the best for callus formation. *D.innoxia* leaf initiated callus on MS medium supplemented with 2mg/l Kin and 0.5-1mg/l 2,4-D. MS medium supplemented with 4mg/l IAA, 1.5mg/l Kin and 0.5mg/l 2,4-D was the best for seed explant of *D.stramonium* and *D.innoxia* as well. 2,4-D alone was better for callus maintenance and growth. UV light increased callus mass. Calli from the two species treated with NaCl senescenced and died. There were no remarkable changes on callus treated with GA3. Insertion of platinum wire in callus tissue enhanced callus growth in *D. stramonium* and *D.innoxia*, resulting in a compact calli for seeds and leaves.

Keyword: *Datura Stramonium, Datura innoxia*, ultra violet, sodium chloride, Gibberellic Acid, Platinum wire

INTRODUCTION

Solanaceae family has a worldwide distribution; it consists of about 98 genera and some 2,700 species **[1]** with a great diversity of morphology and ecology. *Datura* is wide-spread in Sudan, it grows as a weed in northern and central Sudan on a wide range of soils throughout the year **[2]**.

Datura stramonium is herbaceous plant with branched, green to purplish and pubescent stem and about 60-120 cm or more in height and purple in colour, leaves are long-petiolate, simple dentate, jaggedly toothed, The flowers are single, erect, trumpet-shaped and violet (also there is white to creamy flowers), the fruits are large green capsules, spiny, dehiscing *via* four valves and containing numerous reniform dark brown (unripe) to black (ripe) seeds.

Datoura innoxia is an annual shrub by-plant distributed in temperate zones, it is also known as thorn apple, moon flower and Devil's trumpet. The height of *D. innoxia* can reach up to 2m, the stem is semiwoody and green, and the leaves are alternate and ovate in shape. Flowers are white and trumpet-shaped and the fruits are pendulous and covered with many short thorns. The seeds are brown to orange in colour.

Elicitation has been widely used to increase the production or to induce *de novo* synthesis of secondary metabolites in *in vitro* plant cell cultures **[3]**.

MATERIALS AND METHODS

The plant materials (vegetative and reproductive parts) used in this study were collected from different sites in Khartoum State, Sudan. *Datura innoxia* and *Datura stramonium* were collected from Shambat area. The plants were identified, confirmed and authenticated by comparing with an authentic herbarium specimens.

Surface sterilization of seeds:

Normal looking seeds from the two species were selected and surface sterilized with 70% (v/v) ethanol for one minute and then immersed for 15 minute in a 2.5% (v/v) sodium hypochlorite solution or "Clorox" with few drops of a liquid detergent added. Then, the seeds were rinsed 3-4 times with sterile distilled water.

Callus initiation:

The media contained Murashige and Skoog (MS) basal mineral nutrients **[4]** supplemented with 3% sucrose were used as media for culture initiation. For callus initiation the basal MS media were manipulated with auxins {IAA; 2,4-D; NAA}, and cytokinins {BAP; kin}, in different concentrations and combinations from 1mg/l to 12.5mg/l have been used in all experiments. The pH of the media was adjusted to 5.7. For preparation of semi solid media, 0.8% agar was used as the gelling agent.

The sterilization of media and glassware was carried out in an autoclave at 121°C, (15 lb/in2) pressure for 15 minutes before dispensing in culture vessels.

Preparation of explants:

Sterile seedling and leaves were chosen as explants for this study. Seeds were treated with $H_2SO_4$to break the dormancy. Seeds were sterilized as previously described and grown on solidified MS basal medium. Fresh young leaves were collected and washed with tap water then surface sterilized as mentioned before and used for callus initiation.

Callus multiplication and maintenance:

For callus multiplication and maintenance the MS medium was supplemented with different combinations of growth regulators i.e. BAP, IBA,IAA, NAA, BA, Kin or 2,4-D at different concentrations as shown in the table below, in the presence of 3% sucrose and 0.8% agar (pH 5.7) **(Table 1)**.

Media treatment combination
2mg/l BA + 2mg/l NAA
2mg/l 2,4-D+ 2mg/l IAA
2.5mg/l 2,4-D+ 1mg/l IAA
0.5mg/l 2,4-D+ 2mg/l IAA
0.5mg/l 2,4-D+ 1mg/l IAA
1mg/l 2,4-D+ 2mg/l Kin
0.5mg/l 2,4-D+ 1mg/l Kin
0.5mg/l 2,4-D+ 4mg/l IAA+ 1.5mg/l Kin
2.5mg/l BAP + 2mg/l IBA

Exposure to UV-C radiation:

For investigation of the effect of UV-C irradiation on callus, UV-C lamp (Philips UV light 200-280nm) was used as elicitor. The UV light was applied from distances of 20cm for 10 minutes onto the callus cultures the treated calluses were removed and callus morphology and growth parameters were recorded. Callus samples were collected after 8 days.

Treatment with gibberellic acid (GA_3):

About 0.4ml GA_3 (100µm) were aseptically added to 100ml autoclaved MS medium. Callus cultures were transferred to medium supplemented with GA_3 and incubated for 8 days.

NaCl treatment:

Aliquots of 125µm NaCl were added directly to the culture media before autoclaving. Callus cultures were transferred to the medium with NaCl, after 8 days the callus cultures were collected.

Platinum wire application:

Pieces of platinum wire were inserted in a callus culture for 8 days, after that they were removed from the callus.

RESULTS AND DISCUSSION

Seeds of *D. innoxia*, *D. stramonium* were treated with H_2SO_4 and the seed coat was removed before surface sterilization to break dormancy before germination.

Explants from two species of *Datura* were removed under aseptic conditions and transferred aseptically to MS medium supplemented with 3% sucrose, 0.8% agar to solidify the medium and different concentrations from Kin, BAP, BA, IBA, NAA, IAA and 2,4-D were added alone or in different combination.

The results showed that Kin, IAA, 2,4-D were the most effective growth regulators in callus initiation with different concentrations and different combinations.

Tissue culture of *D. stramonium*:

Leaves:

Leaf explants initiated callus about 7 days after culture. The calli at first formed at cut edges of leaves and then expanded throughout the explants. The growth regulators 2,4-D and Kin were the best for callus formation with concentration (0.5&1 mg/l) respectively **(Figure 1)**. From the different concentrations of regulators the combination 4mg/l IAA, 1.5mg/l Kin and 0.5mg/l 2,4-D induced minimum callus production, while 2,4-D alone was better than others for callusgrowth, 2.5mg/l 2,4–D resulted in maximum callus growth **(Figure 2)**. Calli obtained from leaf were white and compact.

[5] reported that the presence of 2, 4-D is necessary for callus induction from leaf explants in *D. stramonium* since the treatments lacking 2,4-D in the media failed in callus formation or produced too little calli and the effect of Kin was not significant.

This indicates that Kin can encourage the effect of 2,4-D on callus formation from leaf explants, but it is not able toform callus solely. Also, **[6]** found that 2.0 mg/l of 2, 4-D was the most effective auxin concentration to induce callus from *D. stramonium* leaf explants. A similar trend was observed in the present study.

In contrast, [**7]** reported that 2,4-D (1.0 mg/l) + Kin (0.25 mg/l) were the best for callus size and weight for potato cv. Diamant.The interaction of both regulators had significant effect on callus induction but low concentration of 2,4-D with high concentration of Kin was found ineffective **[8]**.

Seeds:

The explants in 4mg/l IAA, 1.5mg/l Kin, 0.5mg/l 2,4-D initiated callus earlier than other treatments. The formed calli in this treatment were white to creamy and compact in appearance **(Figure 3a)** which became thoroughly cream within subsequent days, after 2 months, callus formed hypocotyl **(Figure 3b)**. Similar results have been reported by **[9]** in garlic, the callus was induced from leaf explants cultured on MS medium containing l mg/l Kin,1 mg/l IAA and 2 mg/l 2,4-D, the frequency of callus induction being 100%. After 35 days the callus transferred to 0.5mg/l 2,4-D with 1mg/l Kin from media contained Kin+ IAA+2,4-D, sixteen days later a hypocotyl started to appear **(Figure 4a & b)**. Other group of callus initiated from Kin+ IAA+2,4-D and transferred to 2.5mg/l 2,4-D after making some cutting on callus lump, it gave only roots and it became taller with time **(Figure 5a & b)**. While, the callus transferred to 2mg/l 2,4-D

became bigger in size, creamy tobrown and nodular and there was no visible shooting or rooting **(Figure 6a & b)**.

The combination of 1mg/l 2,4-D and 2mg/l Kin showed a good result in callus formation but the increase in callus mass was too slow in comparison to that in 2,4-D, IAA & Kin **(Figure 7a & b)**. The callus shown in Fig. 8 formed from seeds cultured in MS medium supplemented with 2&2.5mg/l 2,4-D only, the callus appeared after 10days and it was small in size and creamy in colour, after days there was no noticeable increase in size **(Figure 8a & b)**. These results are in disagreement with [**5]** who found that the embryo explants in 2mg/l 2,4-D alone enhanced callus formation earlier than other treatments. Also, they reported that addition of Kin to some extent, reduced the effect of 2,4-D on callus formation, so that this negative effect can be increased from 0.5 to 1 mg/l Kin. Although, it was observed that in *Gymnema sylvestris*, to maintain the callus, 2,4-D medium was not effective enough whereas the callus remained healthy and increased in different concentrations of 2,4-D:Kin combination **[10]**.

Tissue culture of *D. innoxia:*

Leaves:

Among all the treatments, those containing 2mg/l Kin in combination with 1 or 0.5mg/l of 2, 4-D initiated callus faster than others. Although, 2.5mg/l 2,4-D formed callus bigger in size **(Figure 9)**.

After 7days of incubation, curling as well as swelling were observed initially from the periphery and later on all the surface of leaf **(Figure 10a)**, the callus formation started from edges and middle of the leaf **(Figure 10b)**.

Formed calli from these treatments were both creamy, friable and bulky **(Figure 9)**, sixty days later the callus started to turn brown but still friable.

The present result was in the same par with **[11]** findings, who reported that among all tested treatments, MS medium supplemented with 3mg/l Kin and 1mg/l 2,4-D was the best medium composition for callogenesis. Also, **[10]** found that the highest efficiency of callus formation from *Gymnemasylvestris* R.Br. (Asclepiadaceae) was observed in the medium containing different concentrations of 2,4-D and Kin. **[12]** reported that, there are only limited research related to tissue culture of *D. innoxia*, and the suitable medium for callus induction from stem explant was NAA (0.5mg/l) alone or in combination with BAP (0.25mg/l) + Kin (0.25mg/l).

Seeds:

Generally, the callus formation results of *D. innoxia* was almost similar to that of *D. stramonium*. The best growth factors combination for callogenesis was 4mg/l

IAA, 1.5mg/l Kin, 0.5mg/l 2,4-D. Also, the callus was white in colour and compact **(Figure 11)**. While, the seeds cultured on MS medium supplemented with 2mg/l and 2.5mg/l 2,4-D produced small sized and white callus.

On the other hand the cultured seed in Kin+ IAA+2,4-D which transferred after 30 days to MS medium supplemented with 2.5mg/l 2,4-D showed a great effect in increasing the callus size (Fig. 12a&b). There is a disagreement between the present result and **[13]** results, who reported that the best callus induction in *Origanum vulgare* was obtained at lower levels of 2,4-D (0.5mg/l or 1mg/l) added to medium. A combination of 1mg/l kin with 0.5mg/l 2,4-D initiated callus in 20days but the callus size was smaller than that formed in Kin+ IAA+2,4-D.

Similar observations were reported by **[14]** who mentioned that the callus induced in Kin supplemented medium was slow growing.

Effect of elicitors on callus morphology:

Effect of UV:

The results showed that UV light increased callus mass among the species, whereas white tissues appeared in *D. stramonium* seed callus and *D. innoxia* leaf callus. There were no changes in callus colour while the texture was compact after 7 days for all species **(Figure 13)**.

[15] and **[16]** reported that UV light stress could decrease plant growth. These results disagree with the present findings.

UV-C damage is not physiologically relevant for plants growing in the sun, short-wavelength (UV-C) radiation from germicidal lamps has often been used to study DNA damage in animals and bacteria, as well as in plants. UV-C has been used because DNA has a strong absorption maximum in the UV-C range (at 260 nm); UV-C photons are highly energetic, and high levels of damage can thus be created quickly. Also, high-output UV-B radiation sources and spectroradiometers are expensive **[17]**.

Effect of NaCl:

Callus from different species after it was transferred to medium with NaCl the colour started to be darker and turned brown in some species **(Figure 14)**, there was no increase in callus size or changes in texture.

Salty environment leads to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm resulting in a reduction of the cytosolic and vacuolar volumes. Salt stress often creates both ionic as well as osmotic stress in plants, resulting in accumulation or decrease of specific secondary metabolites in plants **[18]**.

[11] reported that the results for both species *D.metel* and *D. stramonium* clearly

showed that callus fresh weights decreased by increasing NaCl concentrations.

Effect of gibberellic acid (GA_3):

There were no remarkable changes on the callus treated with GA_3,a brown colour started to appear in callus after 6 days and it was bounded **(Figure 15)**. **[19]** reported that there was a direct relationship between the culture biomass and cell viability: as the biomass increased, the cell viability index increased. They also found that at the high GA_3 concentration (1.0 μM), the culture viability was higher after day 45 and this may indicate that higher concentrations of GA_3 reduce the growth rate and alter the timing of the log phase. Low viability is a sign of reduced metabolic activity associated with mitochondrial function and respiration, which ultimately leads to cell death **[20]**. Cell viability is also linked to stress enhanced cell permeabilization. Thus, continued research on the relationship between root morphology and physiology is important to improve our understanding of root dynamics **[19]**.

Effect of platinum wire:

The insertion of platinum showed good results in increasing of callus size more than UV, the callus was compact for the seeds and leaves **(Figure 16)**. It has been observed that the insertion of a very fine platinum or titanium wire into a disorganized callus of *Helianthus tuberousus* brought about morphological differentiation, whereas control, untreated tissue remained in an undifferentiated state **[21]**.

A green colour appeared in *D. stramonium* leaves and seeds because of increasing in photoperiod **(Figure 15aa, 16a & 16aa)**.

[22] reported that supplementation with abiotic and biotic elicitors to medium cultures lead to either positive or negative development cells or tissues cultures, and the process depends on type and concentration of elicitors, type and source of cultured plant part and the incubation conditions. Also, they reported that the reduction in fresh and dry weight averages of the callus after using abiotic elicitors might be attributed to the stress in the medium inflected by these agents which reflected negatively on cells growth and division, then upon callus growth and suspended cells.

Figure 1: *D. stramonium* callus initiation from sterile leaves on MS medium with 0.5mg/l 2,4-D and 1mg/l Kin. (×1\2)

Figure 2: *D. stramonium* callus initiation from sterile leaves on MS medium with 2.5mg/l 2,4-D only.(×3\4)

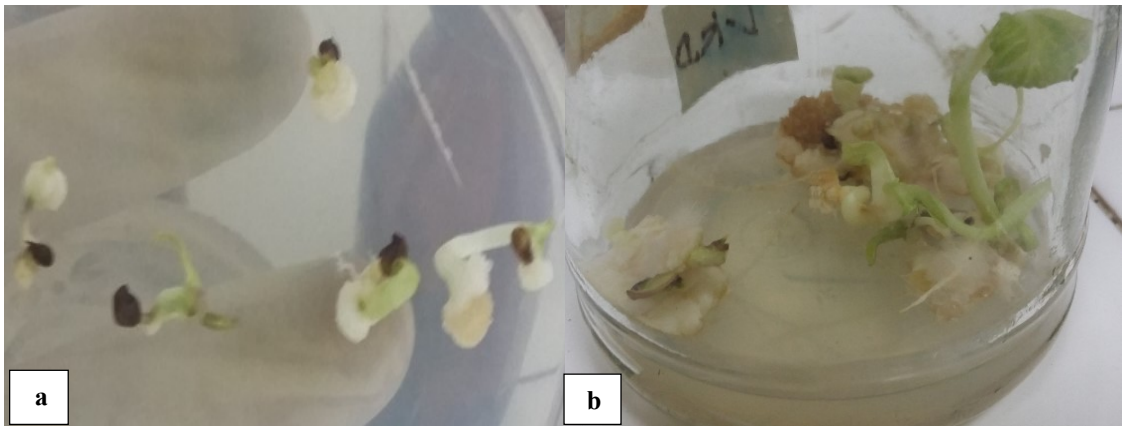

Figure 3: *D. stramonium* seeds cultured on MS medium supplemented with 4mg/l IAA, 1.5mg/l Kin, 0.5 mg/l 2,4-D for a period of :- a: white to creamy callus (30 days old) (×1), b: showing a hypocotyl after 60 days (×1/4).

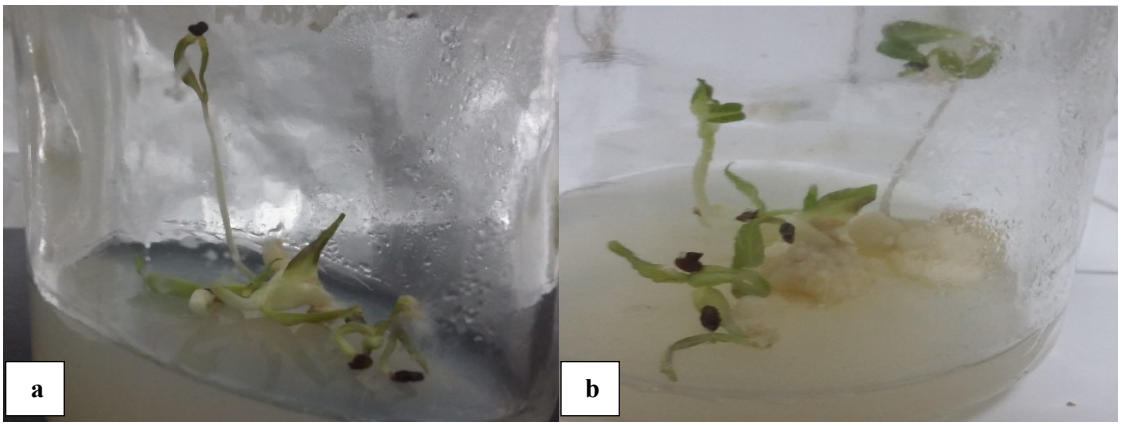

Figure 4: *D. stramonium* seed, callus transferred to MS medium supplemented with 0.5mg/l 2,4-D & 1mg/l kin. a: 16 days on medium with 2,4-D & Kin, b: 20days on the same medium (×1/4).

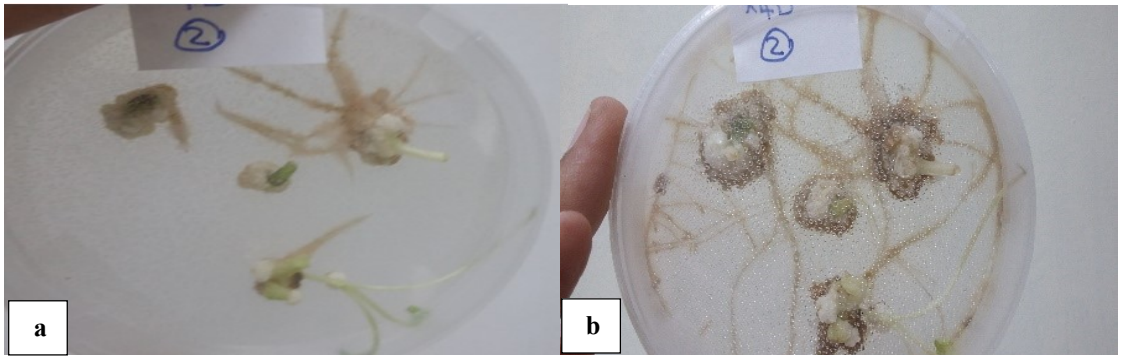

Figure 5: *D. stramonium* seed,callus transferred to MS medium supplemented with 2.5 mg/l 2,4-D. a: 14 days after sub culturing on 2,4-D, b: 22days after sub culturing on 2,4-D, c: 66 days after sub culturing on 2,4-D (×1/2).

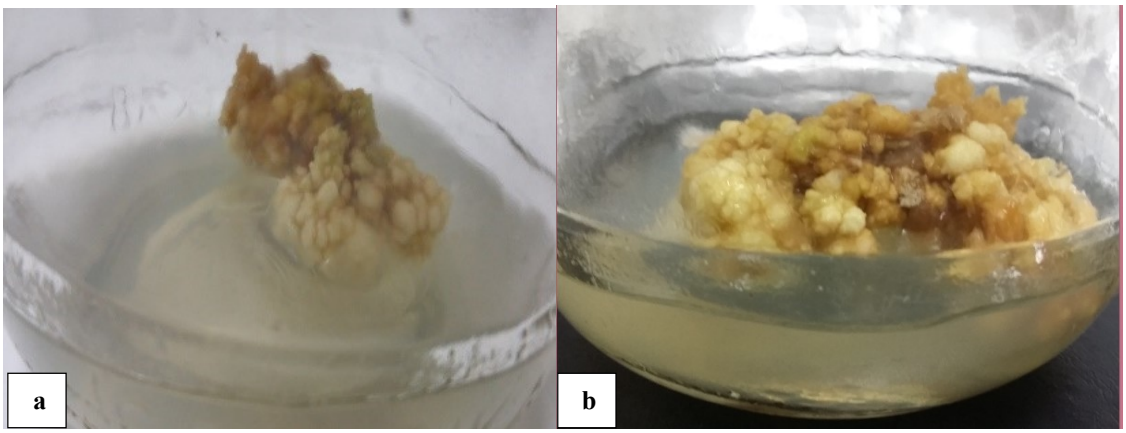

Figure 6: *D. stramonium* seed, callus transferred to MS medium supplemented with 2mg/l 2,4-D. a: nodular creamy callus (15 days) after sub culturing on 2,4-D, b:creamy- brown, nodular compact callus (45days) after sub culturing on 2,4-D (×1/5).

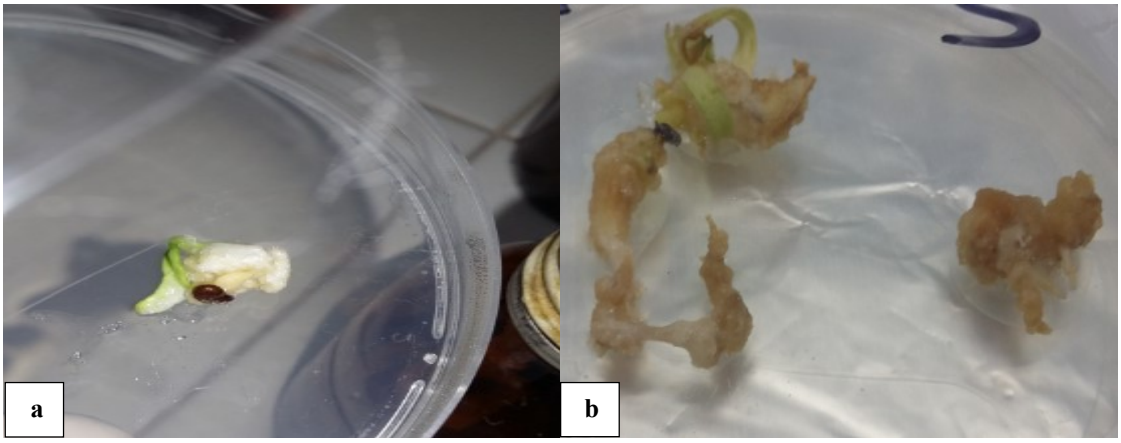

Figure 7: *D. stramonium* seed callus initiation from sterile seedlings on MS medium supplemented with 1mg/l 2,4-D and 2mg/l Kin at different stages of development. a: 16days after inoculation (×4), b: 35days after inoculation (×3).

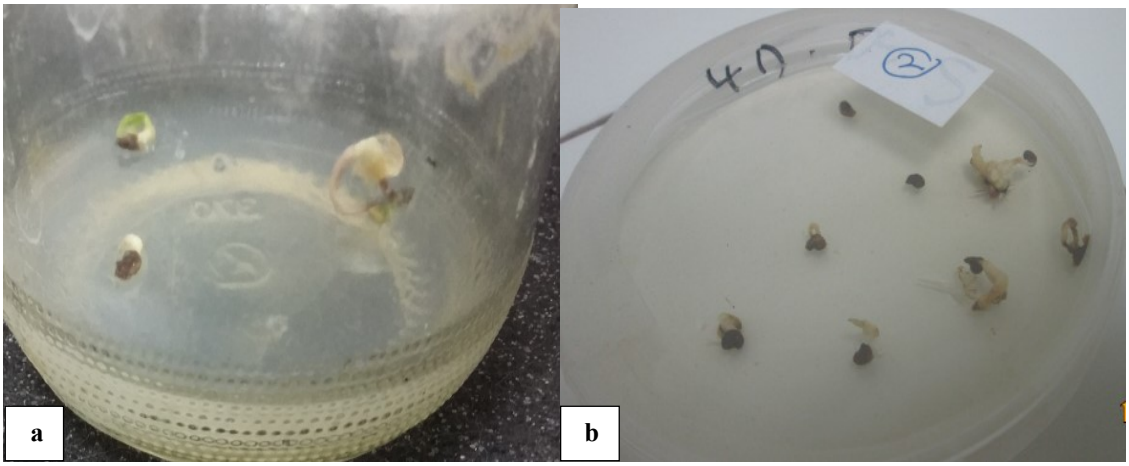

Figure 8: *D. stramonium* seed callus initiation from seeds on MS medium supplemented with 2 & 2.5 mg/l 2,4-D only. a: 35days after inoculation on 2mg/l 2,4-D (×1), b: 150days and 20 days after inoculation on 2.5mg/l 2,4-D (×1/2).

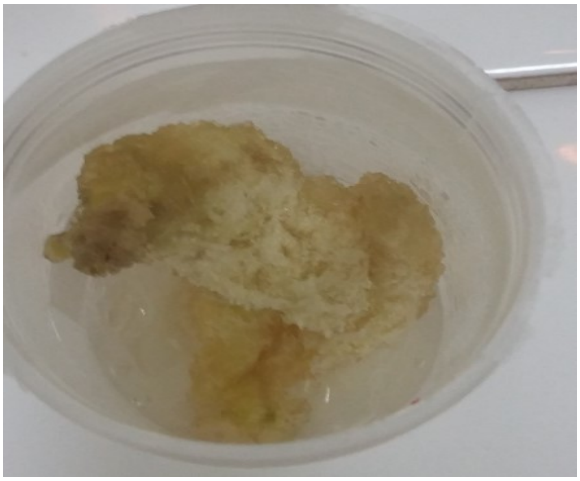

Figure 9: *D. innoxia* leaf cultured on MS medium supplemented with 2.5mg/l 2,4-D for a period of 25days (×1/2).

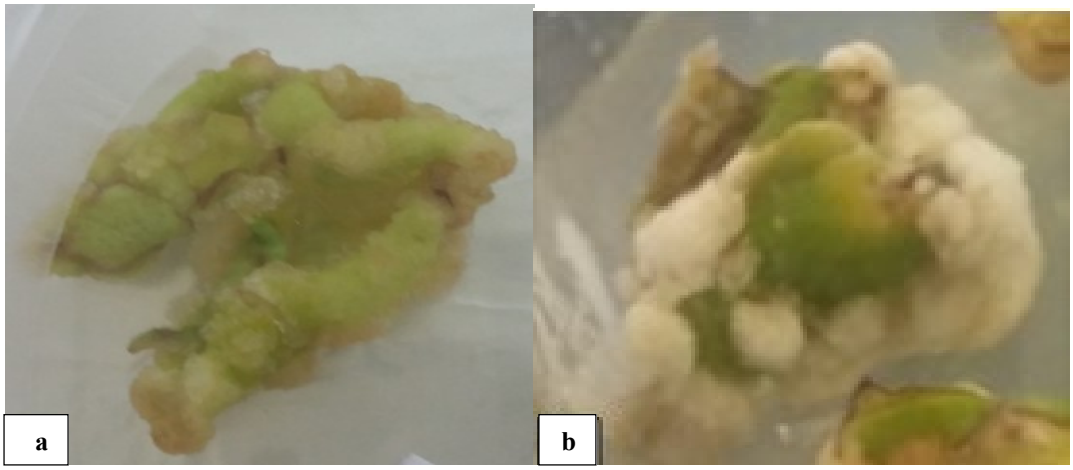

Figure 10: *D. innoxia* callus formation: - a: curling and swelling of leaf (×4), b: started to form callus from edges and middle of the leaf (×4)

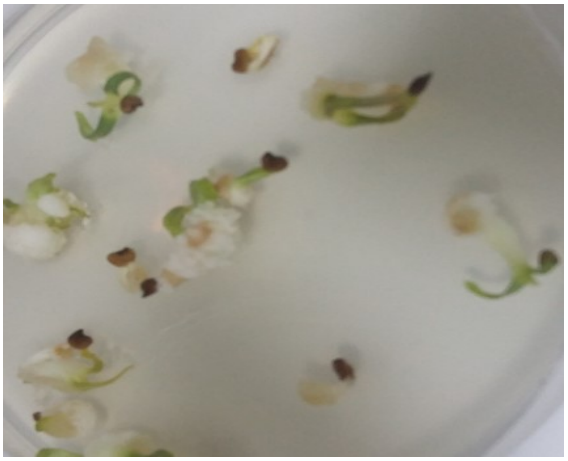

Figure 11: *D. innoxia* seeds,callus induction on MS medium supplemented with 4mg/l IAA, 1.5mg/l Kin, 0.5 mg/l 2,4-D (×1)

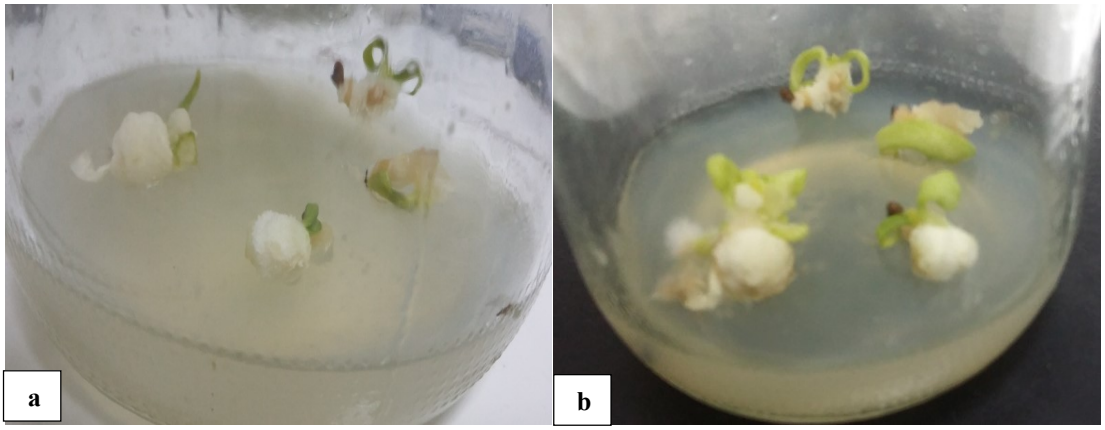

Figure 12: *D. innoxia* callus transferred to MS medium supplemented with 2.5 mg/l 2,4-D only. a: first day after subculture (×1), b: 7days after subculture (×1)

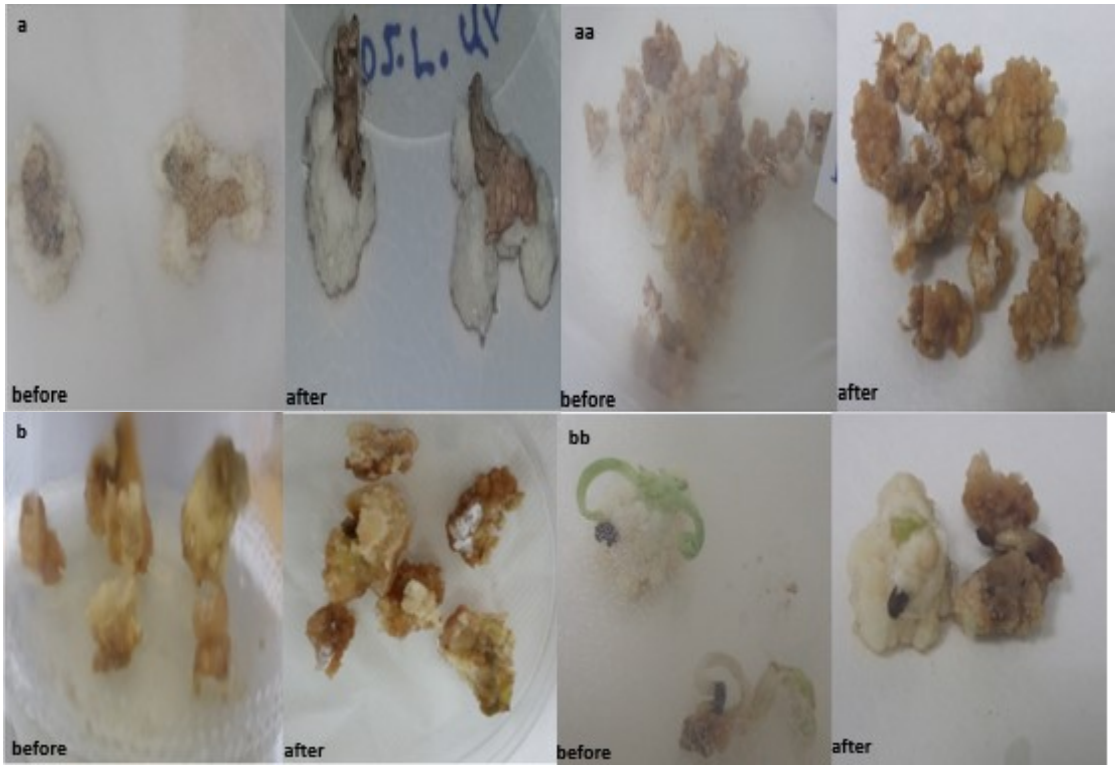

Figure 13: Effect of UV light on callus of *Datura* species

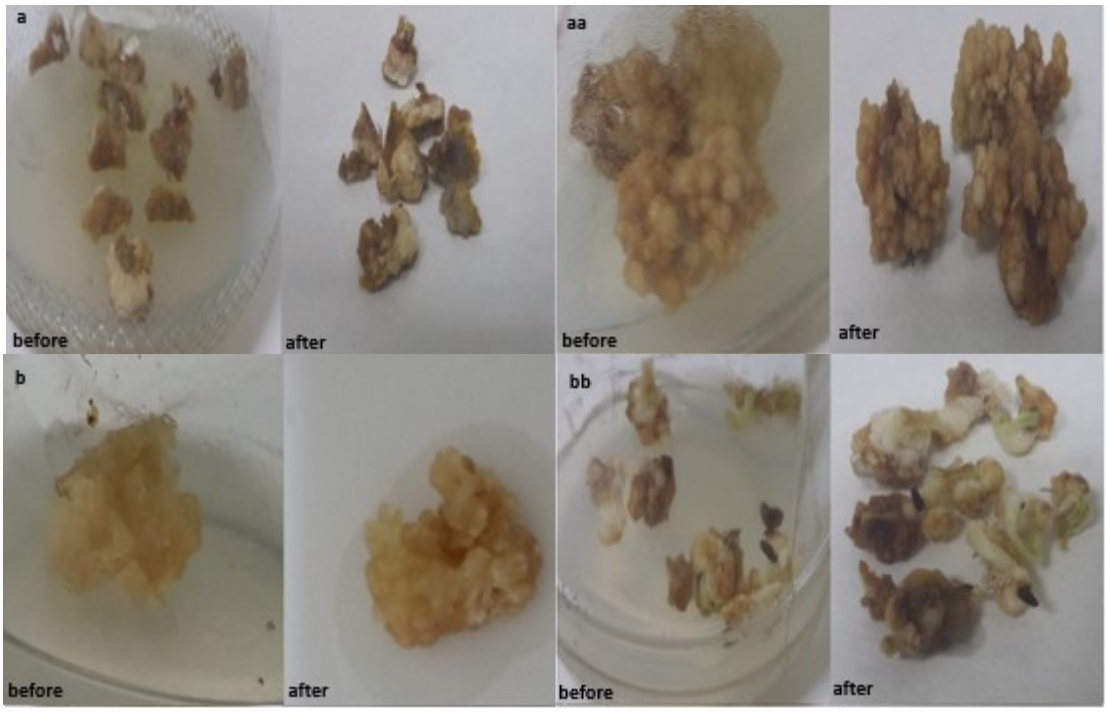

Figure 14: Effect of NaCl on callus of *Datura* and *Solanum* species
a: *D. stramonium* leaves, aa: *D. stramonium* seeds, b: *D. innoxia* leaves, bb: *D. innoxia* seeds. (×1)

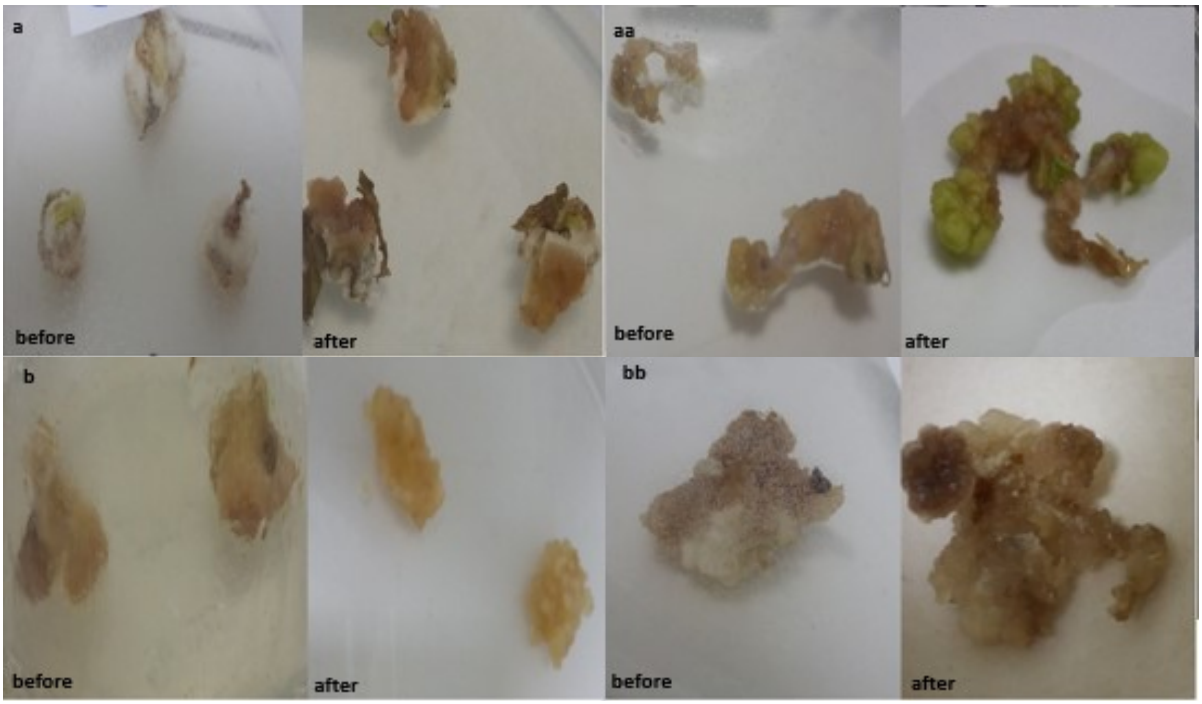

Figure 15: Effect of GA_3 on callus of *Datura* and *Solanum* species

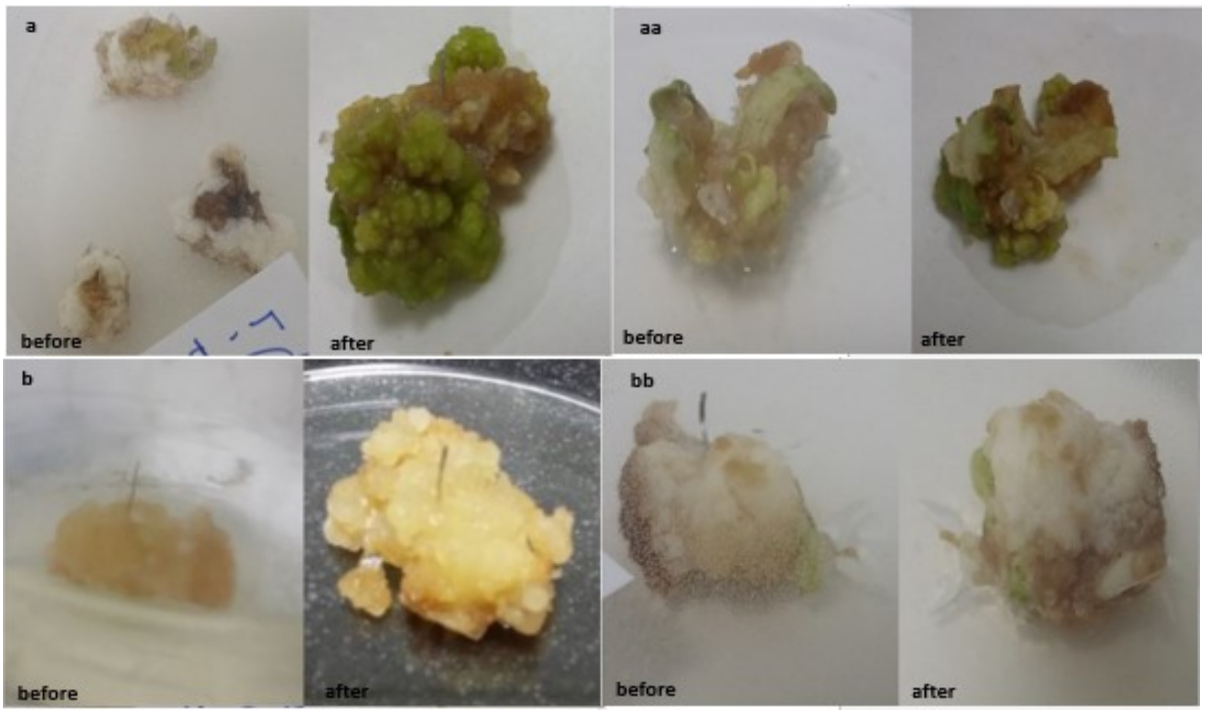

Figure 16: Effect of platinum wire on callus of *Datura* and *Solanum* species
a: *D. stramonium* leaves, aa: *D. stramonium* seeds, b: *D. innoxia* leaves, bb: *D. innoxia* seeds.(×1)

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