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CALLUS INDUCTION AND ADVENTITIOUS SHOOT REGENERATION OF *Hypericum adenotrichum* SPACH

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ABSTRACT

In this study, A protocol to callus formation and adventitious shoot regeneration of *Hypericum adenotrichum* Spach via direct or indirect organogenesis has been described.

Callus induction was carried out by using leaves which were collected from their native environment. The maximum callus induction frequency has been observed on MS medium containing 4 mgL^{-1} BA and 0.2 mgL^{-1} NAA. These calli were not induced to shoot regeneration. Shoot formation was obtained by transferring of the callus from only BA-containing MS medium to MS medium containing 0.5 mgL^{-1} KIN. Maximum shoot number was observed when the callus was transferred from callus induction medium containing 3 mgL^{-1} BA to medium containing 0.5 mgL^{-1} KIN. Direct shoot formation developed on leaf explants of *Hypericum adenotrichum* on MS medium containing KIN alone. The highest shoot number of per explant was observed on MS medium with 1 mgL^{-1} KIN.

Keywords: *Hypericum adenotrichum*, Endemic, Callus induction, Adventitious shoots

Hypericum adenotrichum SPACH'UN KALLUS İNDÜKSİYONU VE ADVENTİF SÜRGÜN REJENERASYONU

ÖZET

Bu çalışmada, *Hypericum adenotrichum* Spach 'un kallus indüksiyonu ve direkt veya dolaylı organogenez yolu ile adventif sürgün rejenerasyonu için bir protokol tanımlanmıştır.

Doğadan toplanan bitkilerin yaprak eksplantlarından kallus oluşumu gözlenmiştir. Maksimum kallus indüksiyon frekansı 4 mgL^{-1} BA ve 0.2 mgL^{-1} NAA ile kombine edilmiş MS ortamında elde edilmiştir. Fakat bu kalluslardan sürgün rejenerasyonu indüklenememiştir. BA'nın tek başına kullanıldığı MS besin ortamlarından elde edilen kalluslar, 0.5 mgL^{-1} KIN içeren MS besi ortamında alt kültüre alındıklarında sürgün oluşumu gerçekleşmiştir. Maksimum sürgün sayısı 3 mgL^{-1} BA içeren kallus indüksiyon ortamlarından alınan kallusların 0.5 mgL^{-1} KIN içeren ortamlara aktarılması ile elde edilmiştir. Direkt sürgün oluşumu ise Kinetinin tek başına kullanıldığı MS besi ortamlarında gerçekleşmiştir. Eksplant başına en yüksek sürgün sayısı 1 mgL^{-1} KIN içeren MS besi ortamında gözlenmiştir.

Anahtar Sözcükler: *Hypericum adenotrichum*, Endemik, Kallus indüksiyonu, Adventif sürgün

1. INTRODUCTION

An obvious increase on medicinal plant production and consumption in the last decades give clues for further development can be seen in future. As known well, only 10% of medicinal plant species are cultivated, remaining 90% are subject of harvest from natural sources these negatively effects natural habitats and their species composition [1]. The lost of natural sources require development of new *ex situ* protection methods and additional measures which support protection studies/ initiations [2]. Cultivation in fields or botanical gardens (for protectional purposes), definition of seed germination conditions, using seeds for mass production, plant tissue culture techniques for micropropagation, seed banks (for storing a great variety of seeds of individual species or more species) and DNA banks are some of the important ex situ protectional methods and activities [3,4].

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Among the medicinal plants, *Hypericum* species have special importance especially in Turkish folk medicine. The most widely used species of *Hypericum* is *Hypericum perforatum* L.. Supply of this species for "botanical industry/market" comes from mostly nature and field production [5]. Harvesting mostly few individuals in a given area, differences in quality among plants from different areas, negatively effected biodiversity and more possibility of contamination or inpurities are driving forces to investigate the most efficient and useful alternative methods for gathering the species at desired amount and quality. At this moment, *in vitro* approaches may serve as one of the most efficient and proper techniques in order to increase knowledge on plant's biology which may give important clues for mass production harvestable plant reserve in an acceptable quality for production of further extracts [6].

For *Hypericum* species, *in vitro* culture techniques was shown as applicable in propagational purposes [7] *In vitro* culture of *H. perforatum* under controlled and standardized conditions was considered as a solution against to complex environmental problems. By using this techniques, *Hypericum* propagation in order to gain plants having similar phytochemical profile, via elimination of negative factors causing production of secondary metabolites by plant in different amounts (changing from individual to individual and among populations) was reported by Wojcik and Podstolski [8].

Hypericum adenotrichum Spach is an endemic species for Turkey. The plant has a capacity for pharmaceutical importance due to the content of hyperforine, pseudohypericin, hypericin, chlorogenic acid, routine, hyperoside, apigenin-7-0- glucoside, quercitrin, quercetin, camphorol, amentoflavone [9]. Among the mentioned components, pseudohypericin and hypericin have antidepressant, antimicrobial, antiviral and antitumoral properties. Exracts of *H. adenotrichum* have anticancer activity [10]. Sarımahmut et. al. [11] investigated genotoxic and apoptotic potential of *H. adenotrichum* Spach. There are also studies for the elicitation of secondary metabolites of *H. adenotrichum* [12-14].

To date, there is no report on the *in vitro* propagation of *Hypericum adenotrichum.* Present study describes a standart protocol to adventitious sh*o*ot regeneration of *H. adenotrichum* via direct and indirect organogenesis.

2. MATERIALS and METHODS

H. adenotrichum plants were collected from Karıncalı Mountain between March and April in active vegetative growth period (1400m. Karacasu, AYDIN; GPS: N 37º 24′ 57,9 ′′, E 28º 20 ′ 25 ′′). The voucher specimens were deposited in the herbarium of Aydın Adnan Menderes Üniversity (AYDN-2396)

In the experiments, fully grown leaves were used as explants. The leaves were kept for about half an hour under running tap water before being sterilized. In explant sterilization, leaves were treated with NaOCl (2.25%) containing two drops of Tween 20 for 5 minutes after kept in 70% (w/v) Ethanol for 30 seconds. Finally, the leaves were rinsed three times with sterile distilled water for 10 min.

Sterilized leaves were cutted into approximately 0.5 cm pairs and cultured in nutrient medium containing different types and concentrations of plant growth regulators to initiate direct or indirect organogenesis process.

In the experiments, Murashige & Skoog medium (MS) [15] was used as a basic medium. The medium was supplemented with 30 gL^{-1} sucrose. pH of medium were adjusted to 5.8 before adding agar (agar-agar, 8 gL^{-1}).

In our experiments, cytokinins such as Benzyl adenine (BA), Kinetin (KIN) (each one 0.1, 0.5, 1, 2, 3, 4 ve 5 mgL^{-1}), Thidiazuran (TDZ; 0.001, 0.01, 0.1, 0.5 ve 1 mgL^{-1}) and auxins such as Naphthalene Acetic Acid (NAA) and 2,4-Dichlorophenoxy Acetic Acid (2,4-D) (0.1, 0.5, 1, 2, 3, 4 ve 5 mgL^{-1}) were used as plant growth regulators. BA, KIN and TDZ were also combined with 0.2, 0.5, 1.0, 2.0 and 4 mgL^{-1}concentrations of NAA. The calli obtained *in vitro* studies were transferred onto MS

medium containing 0.1, 0.5, 1, 2, 3, 4 mgL^{-1} KIN and 0.1, 0.5, 1, 2, 3, 4 mgL^{-1}BA to promote the development of indirect adventitious shoots.

The experiments were repeated twice (5 replicates of 4 explants). The cultures were maintained in a growth chamber at 24 ± 2° C under 16-h light period (40 µE $m^{-2}s^{-1}$). Cultures were subcultured at 4-week intervals. After 30 days of incubation the callus induction frequency was estimated. The frequency of callus induction was calculated according to the following formula.

Callus induction frequency (%): number of explants produced calli / number of leaves cultured X 100. All percent values were subjected to arcsin transformation and shoot numbers were square root transformed before analysis of variance. The data were statistically analysed by using statistical package SPSS version 15.0 in which data subjected to ANOVA. The means were compared using Duncan's Multiple Range Test ($P \leq 0.05$).

3. RESULTS and DISCUSSION

3.1.Callus Formation

Different results were obtained regarding the callus induction frequency in leaf explants of *H. adenotrichum* that was cultured on MS basal medium which was supported with different concentrations of KIN, BA, TDZ, NAA and 2,4-D. Effects of plant growth regulators and concentrations that were used in callus induction medium on the callus induction frequency were found to be statistically significant. Leaf explants of *H. adenotrichum* did not respond to any concentration of NAA, 2,4-D and KIN alone.

Callus induction was provided in the explants on the medium that BA was used alone. Explants have the highest callus induction value on media with 3 mgL^{-1} BA (Figure 1). Callus induction frequency is observed to have fallen on media with BA concentration lower or higher than 3 mgL^{-1}(Table 1).

Callus induction was provided when BA was used together with NAA. The highest callus induction frequency was observed in explants cultivated on MS culture medium with 4 mgL^{-1} BA + 0.2 mgL^{-1} NAA (Figure 2) (Table 1). However, statistically, there was no significant difference in callus induction frequency between 5 mgL^{-1} BA + 0.2 mgL^{-1} NAA and 4 mgL^{-1} BA + 0.2 mgL^{-1} NAA . Due to the effect of NAA in medium with BA and 0.2 mgL^{-1} NAA, an increase in callus induction frequency was observed with respect to increased BA concentration.

Callus induction was not observed in media where KIN was used alone, whereas in cases where it was used with 0.2 mgL^{-1}NAA callus induction was observed due to the effect of NAA. The highest callus induction frequency was observed in media containing 2 mgL^{-1} KIN + 0.2 mgL^{-1} NAA (Figure 3). At concentrations above 2 mgL^{-1} KIN, the callus induction ratio decreased (Table 1).

Table 1. Callus induction frequencies on MS medium with BA alone and BA and KIN in combination with 0.2 mg L^{-1} NAA.

BA (mgL^{-1})	Callus induction frequency (%)*	BA+NAA (mgL^{-1})	Callus induction frequency (%)*	KIN+NAA (mgL^{-1})	Callus induction frequency (%)*
0 (Control)	0^{d}	0+0.2	0^{e}	0+0.2	0^{f}
0.1	0^{d}	0.1 + 0.2	0^{e}	0.1 +0.2	0^{f}
0.5	30^{c}	0.5 +0.2	25^{cd}	0.5 +0.2	75^{ab}
1	30^{c}	1 +0.2	50^{bc}	1 +0.2	75^{ab}
2	60^{b}	2 +0.2	75^{ab}	2 +0.2	**80^{a*}**
3	**80^{a}**	3 +0.2	75^{ab}	3 +0.2	50^{bc}
4	45^{bc}	4 +0.2	**95^{a}**	4 +0.2 N	50^{bc}
5	25^{c}	5 +0.2	90^{a}	5 +0.2	20^{de}

*In each column, the means followed by different letter(s) show significant differences at the $p \leq 0.05$

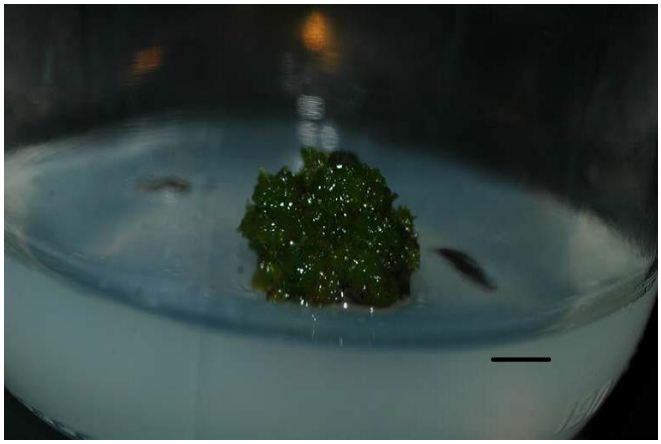

Figure 1. Callus induction on MS medium supplemented with 3 mgL^{-1} BA after 4 weeks of incubation (Bar: 1 cm)

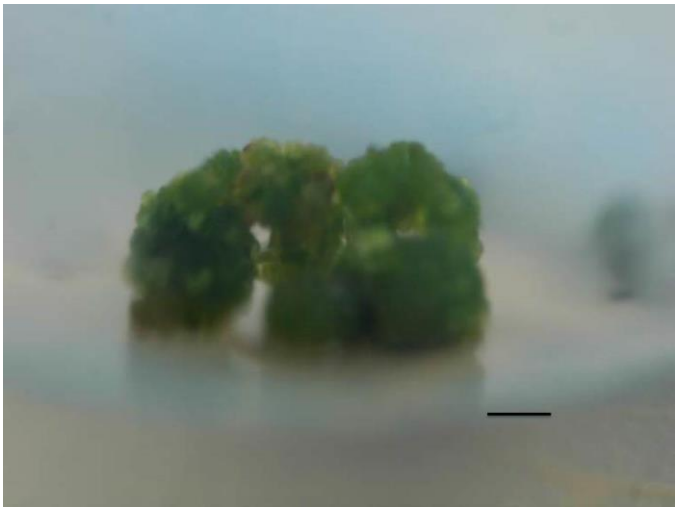

Figure 2. Callus induction on MS medium containing 4 mgL^{-1} BA and 0.2 mgL^{-1} NAA after 4 weeks of incubation (Bar: 1 cm)

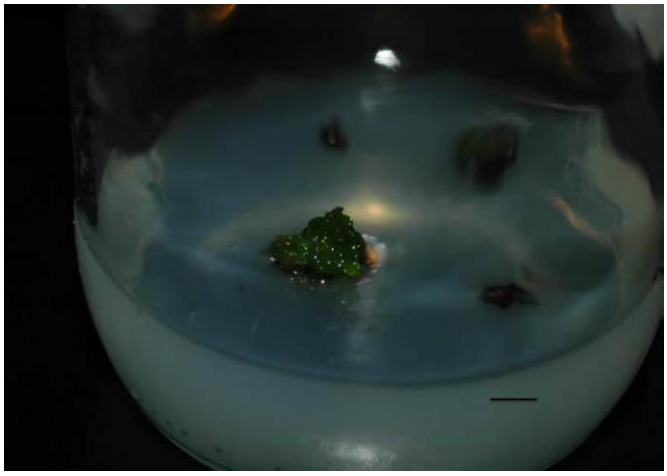

Figure 3. Callus induction on MS medium containing 2 mgL^{-1} KIN and 0.2 mgL^{-1} NAA after 4 weeks of incubation (Bar: 0.5 cm)

Callus induction was observed in the explants which were on MS medium with TDZ was added in different concentrations (Table 2). In explants cultured on medium with TDZ, callus induction frequency is observed to increase based on the TDZ concentration. 1 mgL^{-1} TDZ has the highest callus induction

ratio (Figure 4). Yet, the difference between 1 mgL^{-1} TDZ and 0.5 mgL^{-1} TDZ concentrations is not statistically significant. When the different concentrations of TDZ were used in combination with 0.2, 0.5, 1.0, 2.0 and 4 mgL^{-1} NAA, a callus induction at a significant level was not observed.

Table 2. Effects of TDZ on callus induction frequency

TDZ (mgL^{-1})	Callus induction frequency (%)*
0 (Control)	0^{d*}
0.001	0^{d}
0.01	50^{bc}
0.1	70^{ab}
0.5	80^{a}
1	90^{a*}

*The means followed by different letter(s) show significant differences at the $p \leq 0.05$

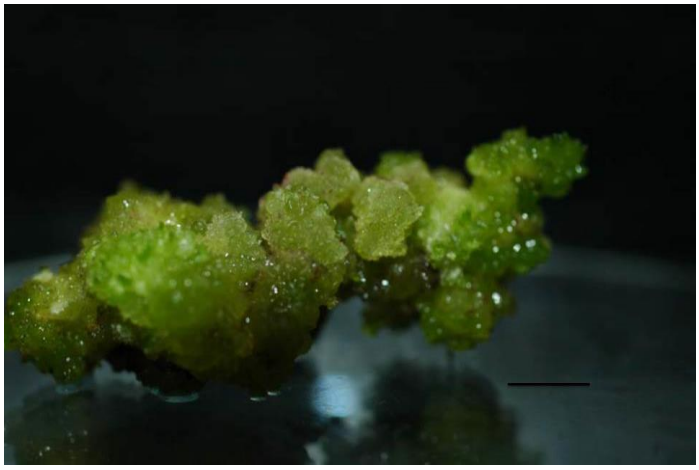

Figure 4. Callus formation on MS medium containing 1 mgL^{-1} TDZ after 4 weeks of incubation (Bar: 1 cm)

When the media that form callus was evaluated, the highest callus induction frequency was observed in explants cultivated on MS culture medium with 4 mgL^{-1} BA + 0.2 mgL^{-1} NAA. (Table 3).

Table 3. Comparison of media with the highest callus induction frequency

Plant growth regulators	Callus induction frequency (%)*
4 mgL^{-1} BA + 0.2 mgL^{-1} NAA	95^{a*}
3 mgL^{-1} BA	80^{b}
2 mgL^{-1}KIN + 0.2 mgL^{-1} NAA	80^{b}
1 mgL^{-1} TDZ	90^{ab}

*In each column, the means followed by different letter(s) show significant differences at the $p \leq 0.05$

Callus tissues have a variety of application areas in plant tissue culture studies. Cell suspension cultures are usually initiated from callus tissues. In addition, callus tissues are used as an intermediate stage for plant regeneration by organogenesis or somatic embryogenesis. The adventitious shoot formation processes by callus tissues are known as indirect organogenesis. [16].

Callus cultures can be used for physiological, biochemical, genetic studies, for studies involving somaclonal variations, for regeneration or reproduction purposes, and for the protection of germplasm [17].

Each callus tissue has not organogenic or embryogenic potency; therefore plant regeneration is not always possible from a callus tissue, and this is mostly because of genetic and epigenetic variations

occurring in callus cells. It is possible to regenerate plants that are phenotypically different from the original plant material using callus tissues. This can be used in breeding programs where some species are restricted in their natural genetic diversity organogenesis [16].

In studies where leaves are used as explant sources in *Hypericum* species, callus formation was obtained on different growth medium which were supported by auxin and cytokinins in different types and concentrations [18- 22]. Calluses were obtained from *H. perforatum* leave explants on medium with 2,4-D and BA or 2,4-D and KIN [18, 21]. *In vitro* culture study performed with *H. mysorence*'s nodal explants, BA in high concentrations (2- 3 mgL^{-1}) promoted callus formation and decreased shoot proliferation [23]. Similar results were also observed for the culture of apical segments of *H. perforatum* [24]. In our study, when BA was used alone or when it was used with NAA, callus formation was obtained from leaf explants, whereas KIN provided callus induction when only used with NAA. No significant callus induction was observed in the combination with 2,4-D. In cases where NAA and 2,4-D are used alone, callus induction has not been achieved. Pretto and Santarem [18] in agreement with our results, reported that the culture of leaf explants did not result in callus formation in the presence of only 2,4-D growth regulator and explants underwent necrosis after 3 weeks of culture in *H. perforatum*. In contrast, Li et al., [19] stated that the best medium for callus induction in *H. perforatum* was the MS medium containing 2,4-D and NAA. In the study completed using *H. brasiliense* nodal explants, various auxin concentrations were tested and the best callus induction was reported on MS or B5 media containing 2,4-D or NAA [25]. The nodal segments of mature *H. perforatum* plants were cultivated in MS medium containing 4.5 μM BA, kinetin, TDZ alone or in combination with 0.05 μM NAA. In addition to the organogenic response; callus formation was observed in explants cultivated with TDZ, NAA or a combination of both (respectively 65.4%, 60.7% and 57.7 [26]. In our study, callus induction was observed from leaf explants only in medium with TDZ. In the combination of TDZ and NAA, no significant callus induction was observed.

3.2. Indirect Adventitious Shoot Regeneration

The callus mass were transferred to shoot regeneration medium containing different plant growth regulators. Shoot induction responses showed difference depending on the type and concentration of growth regulators used in callus induction (Table 4).

The shoot buds were formed by transferring calli obtained from TDZ containing media to shoot regeneration media, but no healthy shoots were obtained from these shoot buds (hyperhydric shoots). The calli obtained from BA + NAA combination could not create shoot in any environment. Shoot buds were obtained from calli promoted by KIN + NAA combination, however a significant level of shoots were not obtained from these buds.

Table 4. Shoot regeneration responses of calluses obtained from different callus induction medium

Plant growth regulators used in callus induction	Adventitious shoot responses
Callus in the medium containing TDZ	Low hyperhydric shoot regeneration
Callus in the medium containing BA and NAA combinations	No shoot regeneration
Callus in the medium containing KIN and NAA combinations	Low shoot regeneration
Callus in the medium containing BA	High shoot regeneration

When the calli obtained from the MS medium with BA only were transferred to the shoot regeneration medium with 0.1, 0..5, 1, 2, 3, and 4 mgL^{-1} KIN, a response was recorded. The highest shoot number per callus mass was obtained in culture on MS growth medium with 0.5 mgL^{-1} KIN. In addition, BA concentrations used in callus induction medium also had significant effects on the number of shoots per callus. The maximum number of shoots per callus was obtained by culturing the calli obtained from 3 mgL^{-1} BA on media containing 0.5 mgL^{-1} KIN (Table 5). The shoots, which have an average

length of approximately 1.0 cm (Figure 5), were transferred to the hormone-free MS medium to prolong their length. In the MS medium, the average shoot length was measured as approximately 3.0 cm after 2 weeks (Figure 6).

Table 5. Shoot induction responses of the callus transferred in the medium containing 0.5 mgL^{-1} Kınetin from media containing different BA concentrations

BA concentrations used in callus induction medium (mgL^{-1})	Shoot number per callus*
0 (Control)	0^{e*}
0.5 mgL^{-1} BA	11.4^{d}
1 mgL^{-1} BA	12.8^{cd}
2 mgL^{-1} BA	18.2^{bc}
3 mgL^{-1} BA	**35.6** ^{a}
4 mgL^{-1} BA	22.8^{b}
5 mgL^{-1} BA	15.3^{cd}

*The means followed by different letter(s) show significant differences at the $p \leq 0.05$

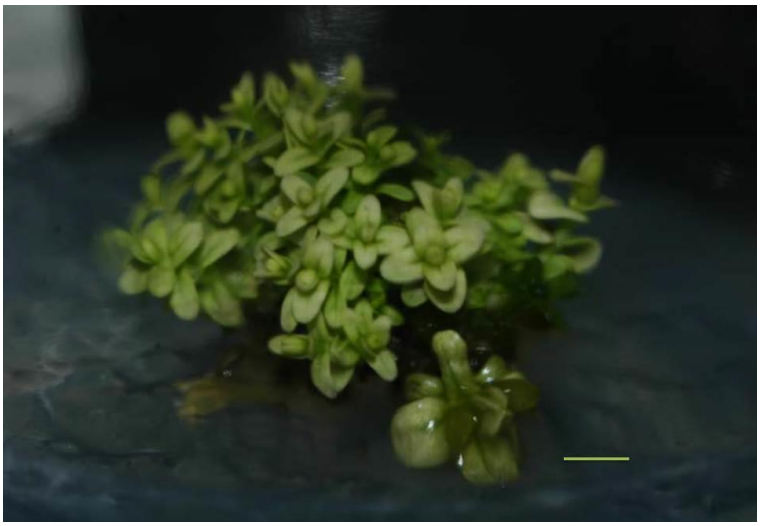

Figure 5. Adventitious shoot on MS medium supplemented with 0.5 mgL^{-1} KIN after 8 weeks of incubation (Bar: 0.2 cm)

Figure 6. Adventitious shoots on without plant growth regulator MS medium after 12 weeks of incubation (Bar: 1 cm)

Pretto and Santarem [18] reported that in calli obtained from *H. perforatum* leaf explants, highest number of shoots per callus was obtained from calli induced on media with 4.6 µM kinetin and 0.45 µM 2,4-D to be cultivated on MS media with 4.4 µM BA. Çırak et al. [27] reported that calli obtained from pupil and body segments in media with BA, 2,4-D and KIN in different concentrations showed an intense shoot progress when cultivated in MS media supported with 1 mgL^{-1}BA for *H. perforatum* and in MS media supported with 2 mgL^{-1} BA. Similar results for *H. perforatum* have also been reported in callus derived from KIN and 2,4-D [28]. In addition, shoot formation from calli from *H. perforatum* was observed on IAA and BA supplemented media. An average of 35 shoots per leaf explant were obtained after a one-month culture period [29]. Studies on *H. perforatum* suggest that BA is more effective as a cytokinin for induction of shoot from callus. In our experiments using leaf explants of *H. adenotrichum*, the highest number of shoots was obtained by transferring the calluses from the 3 mgL^{-1} BA added callus induction medium to the media containing 0.5 mgL^{-1} KIN.

3.3. Direct Adventitious Shoots Regeneration

Leaf explants of *H. adenotrichum* in MS media containing alone KIN first developed shoot initials and then shoots from these initials. Effect of KIN concentrations on shoot number per explant was statistically important (Table 6). The highest shoot number per explant was obtained in MS media supplemented with 1 mgL^{-1} KIN (Figure 7). The shoots, directly induced from leaf explants were 2.8 cm long mainly after 8 weeks of incubation.

Table 6. Averages of direct shoots numbers per explant in MS nutrient medium supplemented with different concentrations of KIN

KIN (mgL^{-1})	Number of direct shoots per explant*
0 (Control)	0^{d*}
0.5	2.8^{c}
1	**7.8^{a}**
2	5.8^{b}
3	3.4^{c}
4	3.2^{c}
5	0^{d}

*In each column, the means followed by different letter(s) show significant differences at the $p \leq 0.05$

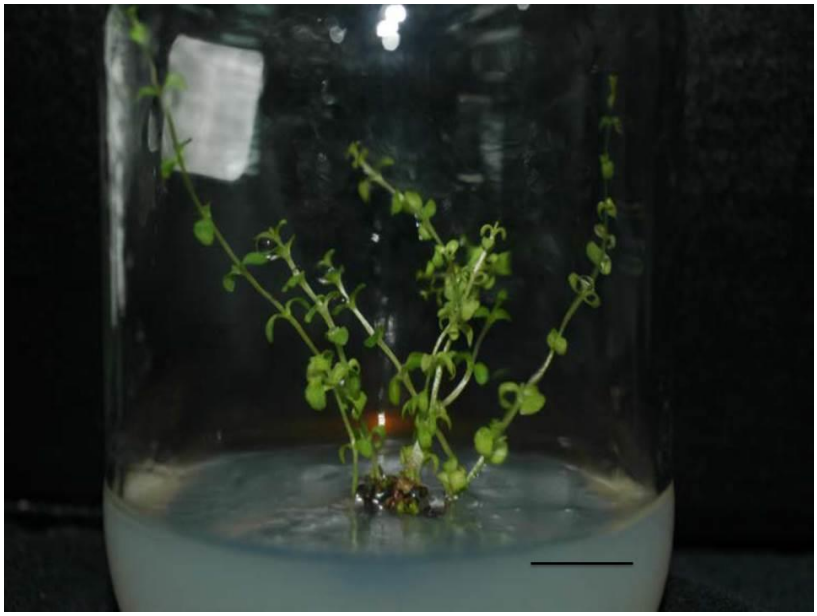

Figure 7. Direct shoot induction on MS medium supplemented with 1 mgL^{-1} KIN (Bar: 1 cm)

The response of the plant growth regulator to direct shoot formation varies in *Hypericum* species. De novo shoot organogenesis in *H. perforatum* was induced by cytokinins (BA, KIN, TDZ) with or without NAA [26]. Shoot numbers induced on nodal explants were changed by type of cytokinin. The highest number of shoots was obtained in the culture medium in which NAA and BA were combined (40.6 shoots/explant). Similar results were obtained for *Hypericum maculatum* [7]. The highest number of shoots was obtained from nodal explants in the presence of BA and NAA in *Hypericum foliosum* [30]. In another study, leaf and internodium explants of *Hypericum bupleuroides* were used and direct shoots were developed in medium containing BA and 2,4 D [31].

4. CONCLUSION

In this study a standart protocol to callus induction and adventitious shoot regeneration of *H. adenotrichum* via direct or indirect organogenesis is presented. The results of this study will lead to micropropagation with *H. adenotrichum*, which is important as a medical plant.

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