



## **Application of Date Seeds Powder as Growth Additive for Callus Induction *in vitro* Using *Vigna radiata* Hypocotyl Seedling Explant**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author SHR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ISAM and SEA managed the analyses of the study. Author SSA managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Date seeds (*Phoenix dactylifera*) are one of the seeds that is not usable and always end to be disposed. The present study describes date seeds as an additive on callus induction *in vitro* from hypocotyl explants of *Vigna radiata*. The objective is to explore the usage of date seed powder as growth additive to promote in plant tissue culture media thereby it can be utilized as fertilizer *in vivo* for sustainable agriculture. 1% concentrations of date seed powder under controlled conditions highly influenced the callus induction. MS media supplied with different auxins is prepared for callus induction. The highest degree of callus weight was observed in MS media supplemented with (5mg/L) 2, 4-D + (0.5 mg/L) Kn (0.437±0.1). MS media supplied with different concentrations of date seed powder + (5mg/L) 2,4-D + (0.5 mg/L) Kn are prepared for callus induction under controlled conditions (16hrs light and 8hrs dark, 3000 lux light intensity, 60% humidity and 25±2<sup>0</sup>c) in a plant growth chamber. MS media supplied with 1% date seed + 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4D gave the highest stimulation of callus growth. Results show that date seed is not

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replacing sucrose as a carbon source, but it acts as a good additive to promote induction callus. Quantitative nutritional analysis of date seed powder was carried out. The results show date seed powder contains a high amount of elements like: Ca (2994.33), K (1712.33), Si (456.33), Mg (687.33), which plays a major role in callus formation.

*Keywords: Plant tissue culture; growth additive; carbon source; tissue culture media; date seed powder.*

## 1. INTRODUCTION

The present study was undertaken to utilize waste date seed material as growth additive supplementation for plant growth. There are number of growth additives efficiency in plant regeneration is tested and well established their usage in plant cell and tissue growth. It is well known in most of the Gulf nations that date seeds are the by-product of date stoning, either for the production of pitted dates or for the manufacture of date paste [1,2]. The date seed is a hard-coated seed, usually oblong, ventrally grooved, with a small embryo. Date pits weigh 0.5 g to 4 g and represent 6 to 20% of the fruit weight depending on maturity, variety, and grade [3,4]. Date seeds are traditionally used for animal feed. They can also be used as a source of oil (which has antioxidant properties valuable in cosmetics), as a coffee substitute, as a raw material for activated carbon, or as an adsorbent for dye-containing waters [5,6]. Date by-products are usually fed to animals during winter, though they can be used at any time of the year [7]. Date seed powder containing various elemental nutritional compositions may serve as a growth additive that is to be used as a supplement in plant tissue culture media. The date fruit analysis has been thoroughly studied and has been in usage for various nutritional importance regular diet. With the advent of biotechnological approaches, culturing plant cells and tissues has turned out to be easier and a boon for conserving and propagating valuable, rare, and endangered medicinal plants [8]. Callus culture offers many advantages as a model system for several biological investigations [9]. Promissory compounds may be useful as a media supplement to develop efficient protocols for in vitro propagation as it favors the shoot formation, callus formation, micropropagation, and various plant breeding applications [8]. An extensive number of complex added substances like coconut water banana mash, peptone, tomato juice, slap nectar, and hamburger concentrate, can be extremely compelling in giving an indistinct blend of natural supplements and development components [10]. Callus serves as

a model system for most of the plant biotechnology-related research since it is a highly proliferative and undifferentiated mass, can be further evaluated and used for wide studies. Through transgenesis, the transgenic cell line carrying transgene can be redifferentiated into a whole organ further regenerated into a transgenic plant with novel characteristics.

## 2. MATERIALS

Tissue culture chamber, laminar airflow, Autoclave, Hot air oven, AAS- Atomic absorption spectrophotometry etc., all the required chemicals used in plant tissue culture media preparation.

## 3. METHOD

### 3.1 Nutritional Analysis of Date Seed

Date seeds of khalas were collected from the date fruit industry and washed with tap water 2-5 times during the month of May 2016. The date seed is dried in a hot air oven at 200 °C for 2 hours. The dried date was ground into fine powder in the mill. The nutritional analysis of date seed powder was analyzed in the ministry of regional municipalities and water resource, Seeb, Muscat, Sultanate of Oman for analysis of date seed including protein content, fat content, fiber content, carbohydrate content, moisture content, ash content, and mineral constituents (Mg, Si, K, Ca, Zn, V, Ni, Mn, Fe, Cu, Cr, Co, and P) through Varian MPX ICP – axial ICP from variant equipped with SP5 autosampler is routinely used for the analysis of cations Ca, Na, Mg, K, Sr, Si, Ba, etc., Varian Grafite Furnace AAS with Hydride generation attachment used routinely for very low levels of trace elements particularly Cd, Pb, Cr, Hg, Anton Parr Microwave digestion unit was used for microwave aided acid digestions of all type of samples.

### 3.2 Effect of Date Seed Powder as an Additive on Rising of Seedling

Healthy rose mong seeds were selected and washed with tap water for 2-5 min, immersed in

5% tween20 for 5min and then washed with distilled water 4-5 times, further seeds were treated with 70% ethanol for 30 Sec followed by 0.1% HgCl<sub>2</sub> for 2 min and finally washed with sterile distilled water for 4-5 times, seeds were inoculated on MS media prepared as a basal medium alone and supplemented with different concentrations of Date seed powder specified in Table 1.

Surface sterilization with 70% ethanol and 0.1%HgCl<sub>2</sub> treatment was carried out aseptically under a laminar airflow cabinet. The cultures after inoculation were labeled and airtight sealed and are incubated under optimum controlled laboratory conditions for 2-4 weeks in a tissue culture chamber.

### 3.3 Effect of Date Seed Powder as an Additive on Callus Induction from Seedling

The hypocotyl seedling explants from 10days old aseptic cultures are used as explant for assessing the effect of date seed powder as an additive on callus induction *invitro*. Prior to inoculation, all the precautionary steps have been taken to establish aseptic conditions. The explant taken from hypocotyl seedling explant is

inoculated on the MS media supplemented with different concentrations of Date seed powder as specified in Table 2.

### 3.4 Explant Preparation

The aseptic seedlings were placed on sterile Petri plates aseptically with the help of forceps and excised into appropriately sized explants from hypocotyl parts with sharp scalpels.

#### 3.4.1 Inoculation

The aseptically prepared explants are inoculated under sterile conditions onto the sterile MS media prepared with different concentrations of date seed powder.

#### 3.4.2 Incubation

The inoculated cultures were covered tightly with the caps and labeled with appropriate titles and are transferred to the culture chamber, and incubated under optimum aseptic culture conditions for a duration of 4-8 weeks. The culture conditions applied are as follows: 16hrs light /8hrs dark, 2000 lux light intensity, 25±2°C temperature, and humidity 65%.

**Table 1. MS basal media with and without date seed powder for rising of aseptic seedling**

| No | Media | Sucrose<br>"Carbon" (%) | Date seed<br>powder (%) | Growth regulator |    |
|----|-------|-------------------------|-------------------------|------------------|----|
|    |       |                         |                         | 2,4D             | Kn |
| 1  | MS    | 3                       |                         | -                | -  |
| 2  | MS    | 3                       | -                       | -                | -  |
| 3  | MS    | -                       |                         | -                | -  |
| 4  | MS    | -                       |                         | -                | -  |

**Table 2. Effect of MS media with date seed powder as an additive on callus induction**

| No | Media | Sucrose<br>"Carbon" (%) | % Date seed<br>powder | Growth regulator |           |
|----|-------|-------------------------|-----------------------|------------------|-----------|
|    |       |                         |                       | 2,4D (mg/L)      | Kn (mg/L) |
| 1  | MS    | 3                       | -                     | -                | -         |
| 2  | MS    | 3                       | -                     | 5                | 0.5       |
| 3  | MS    | -                       | -                     | 5                | 0.5       |
| 4  | MS    | -                       | -                     | -                | -         |
| 5  | MS    | 3                       | 1                     | 5                | 0.5       |
| 6  | MS    | 3                       | 2                     | 5                | 0.5       |
| 7  | MS    | 3                       | 3                     | 5                | 0.5       |
| 8  | MS    | 3                       | 4                     | 5                | 0.5       |
| 9  | MS    | -                       | 1                     | 5                | 0.5       |
| 10 | MS    | -                       | 2                     | 5                | 0.5       |
| 11 | MS    | -                       | 3                     | 5                | 0.5       |
| 12 | MS    | -                       | 4                     | 5                | 0.5       |

### 3.5 Statistical Analysis

Statistical analysis used throughout the study is simple descriptive statistics to evaluate the results mean $\pm$ SD.

## 4. RESULTS AND DISCUSSION

### 4.1 Quantitative Analysis of Date Seed Powder

According to laboratories of the Ministry of Regional Municipalities and Water Resource in the Oman/Seeb region, quantitative nutritional analysis of date seed showed the presence of various food and diet supplements at significant levels, presented in Table 3. The analysis was performed to evaluate fat content, protein content, moisture content, ash content, fiber content, and carbohydrates content. In addition, the elemental analysis was carried out to determine elemental composition like P, Co, Cr, Cu, Fe, Mn, Ni, V, Ca, K, Si, Mg, and Zn.

In the nutritional analysis, it was observed that carbohydrates were found to significant in % (79.74 $\pm$ 0.16) followed by fibers, which are present in moderate % (31.3 $\pm$ 0.15) when compared with the other tested nutrients in date seed powder. The ash content seems to be very low levels. Based on these findings in our objective, we planned to utilize the date seed as a source of carbon in plant tissue culture media to be used as the cheapest source of carbon than regular sugar –sucrose.

Date seed powder analysis for elemental composition also revealed that the presence of high amounts of elements like: Ca (2994.33), k (1712.33), Si (456.33), Mg (687.33). It is assumed that the presence of these elements in date seed powder may help as an additive in tissue culture media for more productivity in agriculture sustainability. Some findings concluded that the presence of potassium and magnesium at higher levels helps in callus proliferation [11]. The current research was aimed to investigate the influence of date seed powder as a carbohydrate source for raising of aseptic seedling and on callus induction from seedling explants.

### 4.2 Effect of Date Seed Powder as an Additive on Raising of the Aseptic Seedling

MS media supplemented with different concentrations of date seed powder alone and in

combination with sucrose to replace as carbon source showed significant influence on seed germination followed by growth. Comparatively, the MS media, supplied with date seed powder 3%, has more strong roots and shoot, which is mostly higher than the control MS media supplemented with 3% sucrose, shown in Fig. 1. and Table 4.

The highest average of shoot length is (11.6  $\pm$ 0.54 cm) observed in MS media supplied with 3% date seed powder+ 3% sucrose. Whereas the MS media supplemented with sucrose alone (11.3 $\pm$ 0.67) and media supplemented with date seed powder alone (11.4 $\pm$ 0.45 cm) and without sucrose and date seed powder (9.66 $\pm$ 0.44cm) was observed. While the root length, leaf count and duration of germination were not influenced by the tested factors. Henceforth it is determined that date seed powder in combination with sucrose in the basal media promotes the growth synergistically instead of as a carbon source. Based on these findings, it is evident that date seed powder can be used as a growth additive in plant tissue culture media but not as a source of carbon. Addition of 15% (v/v) coconut water to the culture media significantly improved callus growth, shoot regenerative capacity, and shoot growth in leaf disk cultures of spinach due to the presence of cytokinin and micronutrients [12].

When 3% of date seed powder is supplied instead of sucrose, it is observed that more strong growth and number of roots, while the same type of media with sucrose, it was clear that more effective root system with more branches has been noticed. But in lengthwise comparatively, the MS media supplemented with 3% sucrose shown maximum length (6.33 $\pm$ 0.35 cm). The results of date seed powder in inducing root system may be due to the high amount of elements. Ca (2994.33ppm), k (1712.33ppm), Si (456.33ppm), Mg (687.33ppm) present in date seed powder, as shown in Table 3.

### 4.3 Effect of Date Seed Powder as an Additive on Callus Induction from the Seedling

Date seed powder as an additive in plant tissue culture media on callus induction showed significant results as additive date seed powder promoted callus formation effectively. The study was carried out with different trials like MS media prepared with and without sucrose in combination with date seed powder at different

concentrations amended with different concentrations and combinations of plant growth regulators.

According to results, there is no callus induction was noticed in MS media supplied with date seed instead of sucrose as carbon source. While Callus is clearly induced in (Fig. 4, Table 5) MS media supplied with different concentrations (1%, 2%, 3% ,4% of date seed powder) along with 3% sucrose supplemented with 0.5 mg/l of Kn +5 mg/l of 2,4D. [13] Found in tissue culture that, higher concentrations of carbon sources (sugars) in the media resulted callus proliferation than shoot differentiation. In our present findings also similar response was showed by hypocotyl explants in presence of both sucrose at 3% and date seed powder at 1-4 % when compared with sucrose alone or date seed powder alone as shown in Table 5.

Results shows that there is a clear effect of date seed powder on callus formation during one week (7 days) of incubation under controlled condition (16hrs light and 8hrs dark, 3000 lux light intensity,60% humidity and  $25\pm 2^{\circ}\text{C}$ ) in a plant growth chamber.

The greater callus degree ( $0.644\pm 0.08$  g) was observed in MS media supplied with 1% date seed + 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4D, whereas the average weight of callus in

control MS media supplied with 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4D is about ( $0.205\pm 0.03$ g). The presence of date seed powder along with MS media supplemented with plant growth regulator exerted more callusing [14]. As per Table 5, it is clear that date seed powder is not replacing sucrose since in the absence of sucrose, the MS media supplemented with date seed powder alone at different concentrations, the degree of callusing was very poor ( $0.054\pm 0.006$  g). Whereas the presence of date seed powder along with MS media amended with sucrose at 3% and different concentrations and combinations of plant growth regulators, the degree of callusing is very high with clear morphological structure as shown in Fig 4. The synergistic additive role of date seed powder in plant tissue culture media may be due to the presence of high concentrations of elements like CA (2994.33), k (1712.33), Si (456.33), Mg (687.33) as shown in Table 3. These elements might have played a synergistic role in callus formation. At the optimum concentration of callus caused callus accumulation and a greater degree of callus formation, in the present findings also at 1% date seed concentration, the similar findings were noticed( $0.644\pm 0.08$  g). As the date seed powder concentration increasing to 2% ( $0.556\pm 0.1$  g), 3 % ( $0.452\pm 0.07$  g), and 4 % ( $0.205\pm 0.05$ ), which means higher calcium concentration negatively

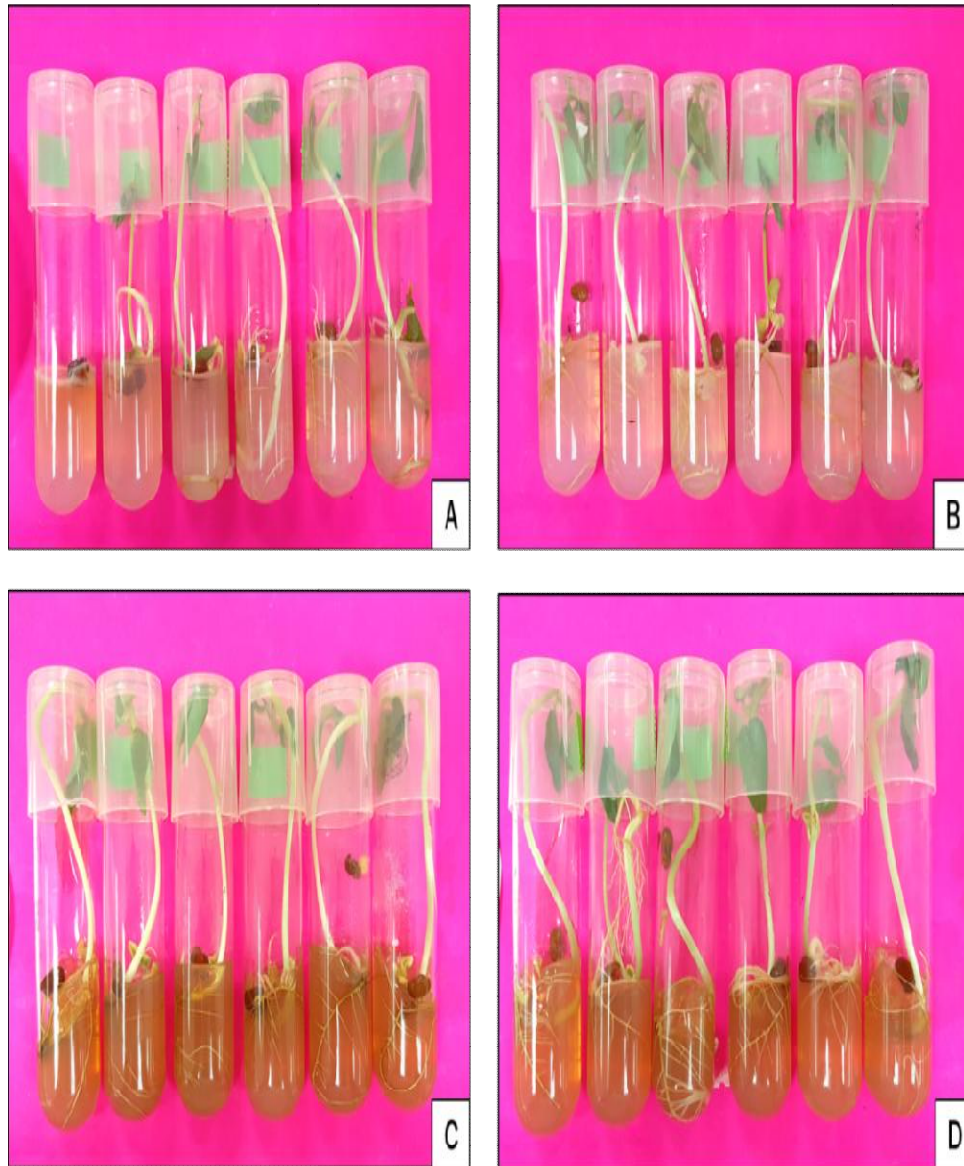
**Table 3. Quantitative nutritional analysis of date seed powder**

| S.no | Content              | Average % | SD of 3 replicate |
|------|----------------------|-----------|-------------------|
| 1    | Fat                  | 9.2       | $9.2\pm 0.13$     |
| 2    | Protein              | 6.1       | $6.1\pm 0.12$     |
| 3    | Ash                  | 0.92      | $0.92\pm 0.01$    |
| 4    | Moisture             | 4.1       | $4.1\pm 0.27$     |
| 5    | Carbohydrates        | 79.74     | $79.74\pm 0.16$   |
| 6    | Fiber                | 31.3      | $31.3\pm 0.15$    |
| 7    | Elements<br>(in ppm) |           |                   |
|      | P                    | 0.25      | $0.25\pm 0.00$    |
|      | Co                   | <0.001    | $<0.001\pm 0.00$  |
|      | Cr                   | 0.34      | $0.34\pm 0.005$   |
|      | Cu                   | 4.37      | $4.37\pm 0.34$    |
|      | Fe                   | 14.83     | $14.83\pm 0.29$   |
|      | Mn                   | 7.18      | $7.18\pm 0.14$    |
|      | Mo                   | 1.81      | $1.81\pm 0.14$    |
|      | Ni                   | 0.52      | $0.52\pm 0.02$    |
|      | V                    | 0.02      | $0.02\pm 0.005$   |
|      | Zn                   | 10.19     | $10.19\pm 0.29$   |
|      | Ca                   | 2994.33   | $2994.38.33\pm$   |
|      | K                    | 1712.33   | $1712.33\pm 18.2$ |
|      | Si                   | 456.33    | $456.33\pm 12.5$  |
|      | Mg                   | 687.33    | $687.33\pm 12.7$  |

Note: All the results explained in the table are the mean  $\pm$ SD of triplicate determination

affected callus growth due to calcium toxicity accumulation. Various studies related to calcium on callus structure also revealed similar findings were at minimum levels, calcium promoted callus formation while at higher levels callus formation is greatly affected [15,16] in addition to Ca, the other elements like K, Si. and Mg might also have played a role directly or indirectly in any of the cellular mechanisms in promoting callus

formation. The presence of Si at higher concentrations in date seed powder also played an important role in plant tissue culture by improving tissue differentiation and morphogenesis in callus formation [17]. In addition to the morphological influence of Si in plant tissue culture, Si also has another beneficial role by protecting the plant cells from metal toxicity and salinity.

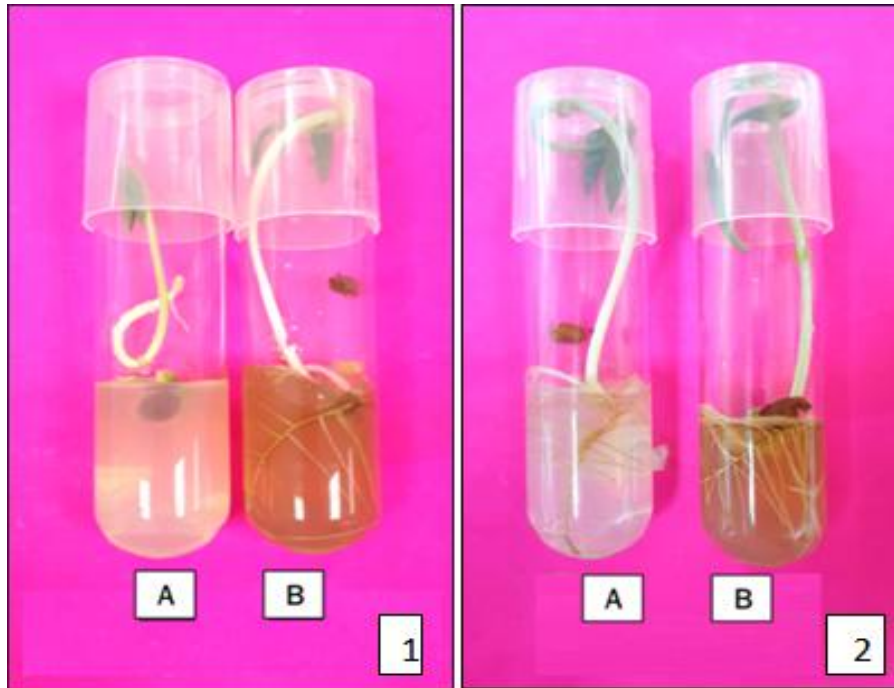


**Fig. 1. Effect of date seed powder as an additive on seed germination. Here A: MS basal media without sucrose, B: MS basal media with 3% sucrose, C: MS basal media without sucrose but with 3% date seed powder, D; MS basal media with 3% sucrose and with 3%date seed powder**

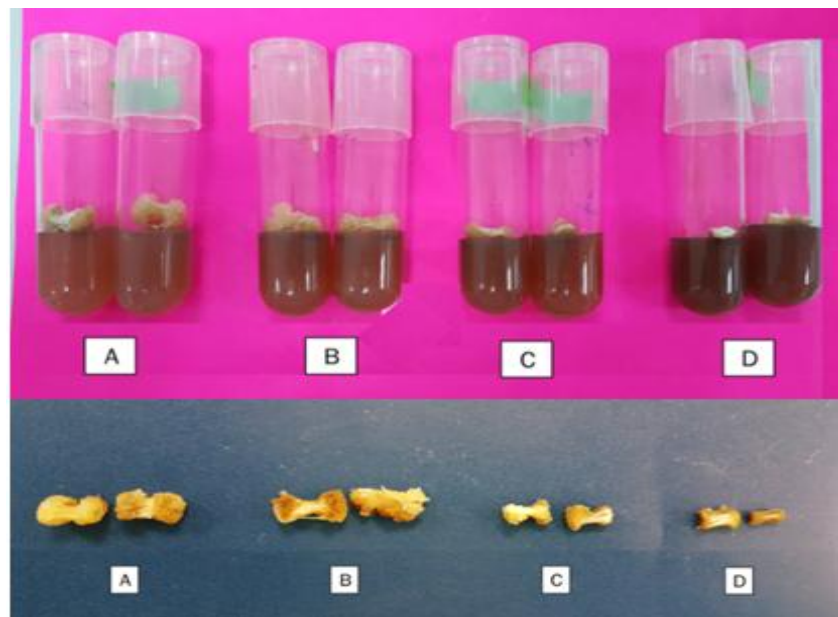
**Table 4. Effect of date seed powder as an additive on seed germination *in vitro* on MS media**

| S. no | Carbo source<br>Sucrose % | Date seed<br>(khalas) | % of germination | shoot length<br>(cm) | root length (cm) | number of<br>leaves | Remarks                         |
|-------|---------------------------|-----------------------|------------------|----------------------|------------------|---------------------|---------------------------------|
| 1     | 3%                        | 3% date seed          | 100%             | 11.6 ±0.54           | 5.6±0.4          | 2                   | More green leaves<br>Thick stem |
| 2     | 3%                        | -                     | 100%             | 11.3±0.67            | 6.33±0.35        | 2                   | Green leaves                    |
| 3     | -                         | 3% date seed          | 100%             | 11.4±0.45            | 6.03±0.53        | 2                   | Green leaves                    |
| 4     | -                         | -                     | 83%              | 9.66±0.44            | 5.7±0.65         | 2                   | Green leaves<br>Thin stem       |

*Note: All Results in the table are the mean ± SED of 10 replicates, and results were recorded after 14 days under incubation*



**Fig. 2. Effect of sucrose alone as a carbohydrate source and in combination with date seed powder on seed germination *invitro* 1-A: MS media with without sucrose,1-B MS media without sucrose replaced with 3% date seed powder, 2-A: MS media with 3% sucrose, 2-B: MS media with 3% of both sucrose and date seed powder**



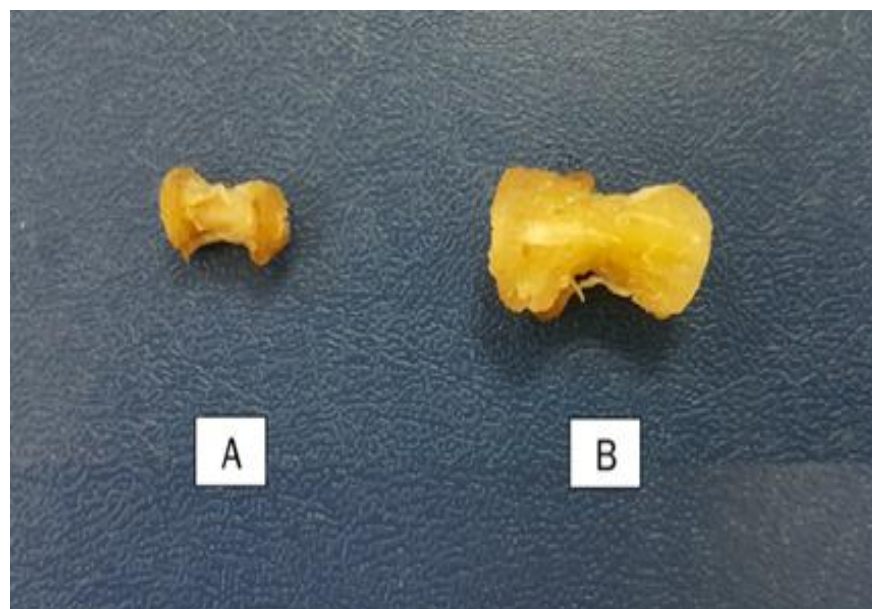
**Fig. 3. Effect of different concentrations of date seed powder amended in MS media with 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4-D. Here A: 1%, B: 2%, C: 3%, D: 4% khalas date seed powder**



**Table 5. Effect of date seed powder as an additive on callus induction from hypocotyl seedling explant of *vigna radiate***

| S.No | Sucrose % | Date seed % | PGRs      |         | Morphology           | color  | Degree of callus weight | Average weight (g) | observation  |
|------|-----------|-------------|-----------|---------|----------------------|--------|-------------------------|--------------------|--|
|      |           |             | 2,4D mg/l | kn mg/l |                      |        |                         |                    |  |
| 1    | 3%        | -           | -         | -       | -                    | -      | 0.135±0.03              | 0.153              | Adventitious roots   |
| 2    | -         | -           | -         | -       | -                    | yellow | 0.053±0.02              | 0.205              | No callus<br>No changes  |
| 3    | 3%        | -           | 5         | 0.5     | Fragile thick callus | -      | 0.205±0.03              | 0.205              | Little callus  |
| 4    | -         | -           | 5         | 0.5     | -                    | yellow | 0.048±0.006             | 0.048              | No callus  |
| 5    | 3%        | 1%          | 5         | 0.5     | Fragile thick callus | -      | 0.644±0.08              | 0.644              | 1% & 2% have greater thick callus while 3% & 4% have less thickness in callus            |
|      |           | 2%          | 5         | 0.5     | Fragile thick callus | yellow | 0.556±0.1               | 0.556              |  |
|      |           | 3%          | 5         | 0.5     | Fragile thick callus | yellow | 0.452±0.07              | 0.452              |  |
|      |           | 4%          | 5         | 0.5     | Fragile thick callus | yellow | 0.205±0.05              | 0.205              |  |
| 6    | -         |             | 5         | 0.5     | -                    | -      | 0.117±0.06              | 0.117              | No results   |
|      |           |             | 5         | 0.5     | -                    | -      | 0.054±0.006             | 0.054              | Though sufficient % of plant growth regulators added in absence of sucrose leads failure |
|      |           |             | 5         | 0.5     | -                    | -      | 0.126±0.07              | 0.126              |  |
|      |           |             | 5         | 0.5     | -                    | -      | 0.054±0.006             | 0.054              |  |

Note: All Results in the table are the mean of 10 replicates and were taken after 4 weeks from inoculation



**Fig. 4. Individual pictures of callus formed from (A) control MS media supplemented with 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4-D (B) MS media supplemented with 1% date seed + 3% sucrose + 0.5 mg/l of Kn +5 mg/l of 2,4-D**

## 5. CONCLUSION

The synergistic role of Date seed powder as a growth additive in plant tissue culture media on callus induction was successfully established. The highest degree of callus weight ( $0.644 \pm 0.08$  g) was observed in MS media amended with 1% date seed powder as growth additive, supplemented with 2,4-D (5mg/L) + Kn(0.5 mg/L). Date seed powder can be considered as a good natural additive for callus growth. As well the study was conducted to determine the role of date seed powder as a carbohydrate instead of sucrose, it showed that it could not be used as a carbohydrate source rather can be used as a growth additive that acts as an additional factor for callus stimulation. The quantitative nutritional and elemental analysis reveals that a high amount of elements present in date seed powder like Ca (2994.33), k (1712.33), Si (456.33), Mg (687.33) may play a major role in callus formation. Therefore the further evaluation is needed in order to use the date seed powder as a nutritive content in plant tissue culture media to make utilize.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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