

Germination and *In Vitro* Propagation of *Gundelia tournefontii* as an Important Medicinal Plant

Mohamad Shatnawi^{1*}, Majid Majdalawi², Wesam Shahrour¹, Taleb R. Abu-Zahra³,
Abdel Rahman Al-Tawaha⁴

¹ Department of Biotechnology, Faculty of Agricultural Technology, Al-Balqa Applied University, 19117 Al-Salt, Jordan

² Faculty of Zarqa, Al-Balqa Applied University, Zarqa, Jordan

³ Department of Plant Production and Protection, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt 19117, Jordan

⁴ Department of Biological Sciences, Al-Hussein Bin Talal University, Maan 71111, Jordan

* Corresponding author's e-mail: abdel-al-tawaha@ahu.edu.jo

ABSTRACT

Medicinal plants are important sources of medical and many other pharmaceutical goods. The conventional propagation scheme is the main means of proliferation and takes a long period of time because of poor germination and also low clonal uniformity. Rapid shoot multiplication of *Gundelia tournefontii* was attained from meristems on Murashige and Skoog (MS) basal medium containing 6-Benzyladenine (BAP) or kinetin, with the adding of 30 g/L sucrose. The highest number of new microshoots per explant (9.2) was attained on the MS medium enhanced with BAP and 0.05 mg/L IBA. After 13 to 14 days, microshoots started rooting on the MS medium enhanced either Indole-3-butyric acid (IBA), Indole -3-acetic acid (IAA), or Naphthalene acetic acid (NAA) with the addition of 30 g/L sucrose. Using 100 to 300 ppm Gibberline GA₃ resulted in an increase in germination percentage when soaking with GA₃ for 12 hours. Gamma radiation had a significant effect on the growth of the *in vitro* explants. Gamma radiation had a significant effect on the germination percentage of *G. tournefontii* seeds. The *in vitro* propagation plant of *G. tournefontii* plants could be used for the marketable clonal propagation of this important species or for future studies.

Keywords: Gibberline, *Gundelia tournefontii*, medicinal plant, proliferation, root formation.

INTRODUCTION

Jordan has been rich in a variety of medicinal plants due to its position. Recently, some studies have been conducted on Jordan flora with special concern on commonly used medicinal plants (Ali-Shtayeh et al., 2000; Jamshidi-Kia et al., 2018). Medicinal plants have attained increasing significance in recent years. Most people in developing countries are dependent on old-fashioned medication for the use of plant extracts (Mohammed, 2019).

Gundelia tournefontii L. (Compositae family) or Akub is a small spiny aromatic perennial herb thistle. *G. tournefontii* is an important medicinal

plant that belongs to the Asteraceae (Compositae) family which grows all over Jordan (Oweis et al., 2004; Shibli et al., 2009; Alimoradi et al., 2019). The *G. tournefontii* plants have a thick perennial rootstock, from which new growth ascends each season. *G. tournefontii* is a “spiny thistle-like perennial plant about 60 cm height, and its stems branch from the base and almost hairless” (Amer et al., 2020). The usage of this plant is possibly quite old. All plant parts (stems, flower, leaves and seeds) can be used as food. *G. tournefontii* grows in mountain slopes and foothills. This plant has a wide range of nutrition and is used in vegetarian dishes for its old-style remedial and nutritive goods (Lev-Yadun and Abbo, 1999).

In traditional medication, the plant shoot is a well-thought-out hepatoprotective and blood purifier (Asadi-Samani et al., 2013; Amer et al., 2020) The *G. tournefortii* extract was reported to decrease the plasma lipid level and contains phenolic compounds such as quercetin with powerful antioxidant effects (Apak et al., 2007; Alimoradi et al., 2017). The edible parts of *G. tournefortii* are a rich source of minerals and vitamins C, B, and A and have been therapeutically used in the old medication system (Karimi et al., 2004; Askari et al., 2008; Tabibian et al., 2013).

A lot of work is required to have it presented for use by the Jordanian people. Land cultivation and overgrazing are the major threats to its existence for consumption (Shibli et al., 2009; Yazdanshenas et al., 2016). Over-collection of *G. tournefortii* threatened the plant resulting in rapid depletion of these genetic resources; therefore, the plant needs protection [Oweis et al., 2004; Shibli et al., 2009]. This can be attained by founding mother plants in the greenhouse, field, or by tissue culture. The seeds of *G. tournefortii* showed a low germination rate, short viability, as well as susceptibility to diseases and pests under moist conditions. Moreover, the agriculture industry is looking for new native varieties of plants with stress tolerances and good properties for the commercial markets. *G. tournefortii* is considered a valuable cash crop that could possibly grow well in degraded land in commercial plantations. The growing request forced on the land and water resources in Jordan and population pressure calls for re-assessment of marginal and under-utilized plantations of *G. tournefortii* (Oweis et al., 2004; Shibli et al., 2009).

In vitro culture can be considered as a rapid, useful, and economic tool for evaluating plant tolerance, specifically in the species with long reproductive cycles (Shibli et al., 2009; Shatnawi et al., 2019). Micropropagation of shoots is easy *in vitro* and to establish plantations in dry or saline soils the selected plant material from drought or salt-stressed cultures can be used. Tissue culture could be a substitute method for the quick propagation of the *G. tournefortii* plants. *In vitro* propagation schemes produce uniform plants with a great scale in a short time (Shatnawi et al., 2019). To the authors' knowledge up to date, there have been no previous published reports on domestication and *in vitro* multiplication of *G. tournefortii*. Therefore, by developing such cash crops, we would not only be creating new economically viable crops for the regions, but also study important healthy plants.

MATERIAL AND METHODS

Seed germination

The seeds of *Gundelia tournefortii* used in this investigation were collected from the middle region of Jordan, mainly from the Al salt Mountain, about 700-800 meters above sea level, (2°02'21.01" N Latitude and 35°43'37.99" Longitude). The seeds were extracted from the dried inflorescences and kept at 4 °C in the refrigerator. Initial estimates of seed viability were determined on four representative samples (replicates) of 40 seeds each using the tetrazolium method.

Greenhouse experiments

In a greenhouse experiment, dry seeds were soaked in GA₃ at 0.0, 200, 400, and 600 mg/L for 12 h and sown in 1 Peat: 1 Perlite mixture in polystyrene trays (20 seeds/treatment) under intermittent mist. Germination percentage was recorded after four week interval. The plants were maintained under greenhouse conditions (24 ± 4 °C). The seeds were considered germinated when a radical emerged and reached around 5 cm. Finally, the germinated seeds for different treatments were planted on the mixture in pots of 1 peat: 1 perlite 1: soil (clay loam) and transferred to the greenhouse (at 24 ± 2 °C day at 15 ± 5 °C night) for further growth and development.

Seed treatment with gamma irradiation

Gamma irradiation was sourced from Cobalt-60 rods at the Jordanian Nuclear Science and Technology Organization (Amman Jordan). The 24 cobalt rods emitted radiation at a measured dose rate of 2.45 Gy/minutes. One densimeter was placed on the outside of the circle of containers, and one on the inside to obtain an exact measurement of radiation emission. The cardboard container was placed in a large cylindrical airtight metal container, which was lowered into the pool containing the cobalt source for a calculated period of time.

The four doses of gamma irradiation were 0, 15, 30, and 46.3 Gy, where the 46.3 Gy treatment was measured with the densimeters and 15 and 30 Gy were obtained by calculations. The vessels were placed 1 minute from the radiation source and radiation administered in a 30 x 30 m field at a dose of 600 monitor Units/minute (6.06 Gy/minute), 100 SSD, 180° gantry, and ColliRTN

0.0°. Radiation was focused at 30 mm from the base of the Perspex container, which was the approximate height of the seeds. The total monitor units (MU) per treatment, are as follows:

- 0.0 Gy = 0 MU
- 15 Gy = 1486 MU
- 30 Gy = 2971 MU
- 46.3 Gy = 4584 MU

Within 2.5 hours of the radiation exposure, all explants had been removed from their container and placed onto a fresh medium. They were then incubated in the growth chamber and assessed for growth.

***In vitro* establishment**

G. tournefortii seeds were sterilized by using 4% NaOCl for 10 minutes, then implanted in 70% ethanol with shaking for 1 minute; afterwards, they were washed with sterile distilled water for 5 minutes four times. After sterilization, the seeds were then washed for three periods under a laminar flow cabinet using sterile water. The seeds were germinated firstly on agar water media, then shoots were cultivated on Murashige and Skoog (MS) (Murashige and Skoog, 1962). The medium pH was adjusted to 5.8. Afterwards, 80 mL of medium was dispensed in each 250 mL flask. The media were autoclaved for 20 minutes at 121 °C. Microshoots were incubated in the growth chamber at 24 ± 2 °C with a 16 h photoperiod and photosynthetic photon flux density (PPFD) of $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ supplied by cool white fluorescent lamps”.

Influence of cytokinins on shoot propagation

Before any experiment, explants were subcultured onto the MS medium with growth regulators free, to avoid any excess residue of BAP. Single microshoots (10–12 mm) in length were placed onto the MS medium containing one of the following treatment treatments. The MS medium was added with different concentrations of BAP or kinetin at 0.0, 0.5, 1.0, 1.5 or 2.0 mg/L with or without the addition of 0.05 mg/L IBA. Following six weeks in culture, the shoot number and shoot length were recorded.

Influence of auxins on formation of root

The impact of indole-3-butyric acid (IBA), indole acetic acid (IAA) and naphthalene acetic acid (NAA) were assessed at a concentration of 0.0, 0.5,

1.0, 1.5, 2.0 or 3.0 mg/L. The number of explants, shoot length, root number, root length and rooting percentage were recorded after six weeks.

Statistical analysis

The treatments in each experiment were arranged in a Completely Randomized Design (CRD) and each treatment was repeated three times. Analysis of variance (ANOVA) was used and mean separation was tested at a 0.05 probability level, according to Tukey’s HSD. The data were statistically analyzed using SPSS analysis system” (SPSS, 2017).

RESULTS AND ANALYSIS

Establishment of a tissue culture plants

One of the major difficulties perceived at the establishment stage of the *in vitro* culture was the bacterial and fungal contamination of the explants, especially in *G. tournefortii*. Moreover, the current study on *G. tournefortii* showed that there were some difficulties in sterilizing the seeds of *G. tournefortii*, as the seeds release a high amount of phenolic compound in the medium. The second problem was the vitrification of the explants. In this study 85% of the inoculated seeds were free of visible contamination after sterilization. After 3 weeks of germination, seedlings reached 1.5–2.5 cm in length. Then, the seeds were separated from their roots and used as primary explants to initiate *in vitro* *G. tournefortii* stock.

***In vitro* shoot multiplication**

The adding of different BAP and kinetin significantly increased the number of shoots (Table 1). The *in vitro* *G. tournefortii* culture had shown high response in the MS medium provided with 1.5 mg/L BAP (9.2 microshoot/explants). Increasing BAP from 0.5 to 1.5 positively influenced the number of new microshoots produced from each explant. In this study, it was found that BAP produced higher shoot proliferation compared with kinetin (Table 1). In a medium containing BAP in different concentrations, an average shoot length increased significantly. Maximum shoot length (55 mm) obtained in medium contains 2.0 mg/L BAP. Maximum shoot formation was induced by a combination of 0.05 IBA and BAP or kinetin. In a medium encompassing 0.5 mg/L kinetin,

Table 1. Impact of BAP or kinetin on the number of new shoots, shoot length of *in vitro* grown *G. tournefortii* after six weeks of culture

Growth regulators (mg/L)		Number of new shoot/explants	Length of shoot (mm)	Growth regulators (mg/L)		Number of new shoot/explants	Length of shoot (mm)
BAP	IBA			PBA	IBA		
0.0	0.0	1.5e	33c	0.0	0.05	1.5d	32c
0.5	0.0	3.7c	35c	0.5	0.05	5.1b	34c
1.0	0.0	3.6c	44b	1.0	0.05	5.3b	37c
1.5	0.0	4.8a	44b	1.5	0.05	9.2a	48b
2.0	0.0	3.9b	55a	2.0	0.05	4.9b	55a
Kinetin	IBA			Kinetin	IBA		
0.5	0.0	1.4e	34c	0.5	0.05	1.9d	33c
1.0	0.0	2.6d	36c	1.0	0.05	3.4c	48b
1.5	0.0	3.3c	42b	1.5	0.05	3.7c	47b
2.0	0.0	3.3c	53a	2.0	0.05	3.8c	48b

an average shoot per plant was 4.8, with 44 mm shoot length. Using diverse concentrations of BA or kinetin resulted in a different number of new microshoots and shoots (Table 1).

Soft, friable, and white callus were produced at the base of induced shoots when BAP and kinetin were supplied. A rosette of shoots with small leaves was developed, rather than elongated normal shoots on the medium supplied with BAP without any root induction (Table 1). The addition of BAP, or kinetin had an overall increased number

of new shoots/explants and increased shoot length (Table 1). Kinetin proved to be less active in comparison to BAP in the promotion of shoot formation. More than 1.0 mg/L either of BAP or kinetin caused a significantly increased shoot formation.

Root formation

Microshoots were cut and cultured on the MS medium added with various amounts of IBA, IAA, and NAA for rooting. *In vitro G. tournefortii*

Table 2. Impact of IBA, IAA, and NAA on the number of shoots, shoot length, number of root and roots length of *in vitro* growth *G. tournefortii* after six week growth periods

Growth regulator (mg/L)	Number of shoots/explant	Shoot length (mm)	Number of roots/explant	Root length (mm)	Root formation (%)
IBA					
0.0	1.30a	19.8a	0.0a	0.0a	0.0
0.5	2.40ab	50.7c	7.9b	50.5b	90
1.0	1.40ab	50.7c	12.9c	48.4b	95
1.5	1.45ab	60.8d	16.1d	50.8b	100
2.0	1.45ab	52.8c	16.7d	47.3b	100
3.0	1.45ab	61.4d	8.8b	47.9b	100
IAA					
0.5	1.50ab	43.3b	8.10b	53.5c	95
1.0	1.50ab	50.4c	8.30b	60.9c	90
1.5	1.48ab	50.7c	8.10b	61.7c	95
2.0	1.30a	39.4b	8.20b	60.7c	90
3.0	1.50ab	39.5 _b	8.25b	61.8c	100
NAA					
0.5	1.40ab	39.5b	8.20b	43.5b	80
1.0	1.4ab	50.5c	7.90b	45.2b	85
1.5	1.50ab	60.5d	8.20b	55.8d	90
2.0	1.45ab	39.5b	1.30b	56.7d	90
3.0	1.30a	39.3b	1.35b	45.8b	100

were magnificently rooted on the MS medium complemented with different concentrations of IBA, IAA, and NAA (Tables 2). Root initiation was first observed after 12 days with IBA, IAA, or NAA auxin. Moreover, at the bases of shoots root and callus were attained. There were no variances in the proportion of shoots emerging roots, between the three auxins used.

Maximum root number (16.1) was obtained when IBA was used at 2.0 mg/L, with 47.3 mm root length. Root length significantly increased along with the concentration of IBA, IAA, or NAA. Shoot length was significantly increased with IAA, compared with the control (Table 2). Rooting was poor on hormone-free media. On the MS medium having IBA, IAA or NAA, 80% to 100% of *G. tournefortii* micrshoots were successfully rooted. Maximum root induction (100%) was attained on the medium fortified with 1.5-3.0 mg/L IBA. Maximum root

formation on the medium supplemented with 3.0 mg/L IAA or NAA was 100%. At the higher concentration (2.0-3.0 mg/L) of IBA, IAA, or NAA, the percentage of rooting was increased, but callus formation was noticed at the base of the shoot to be large (Table 2).

Seed germination

A highly significant effect of the GA₃ concentrations on the *G. tournefortii* germination is shown in Figure (1). The highest germination percentage was at 200 ppm GA₃ (83.3%). No significant differences between dry seeds and those soaked in water 30% germination percentage. Using 200 ppm of GA₃ resulted in a drastically increased germination percentage (up to 90%) when soaking with 400 GA₃ for 12 h. Germination percentage increased when using GA₃ up to 600 ppm (Figure 1)

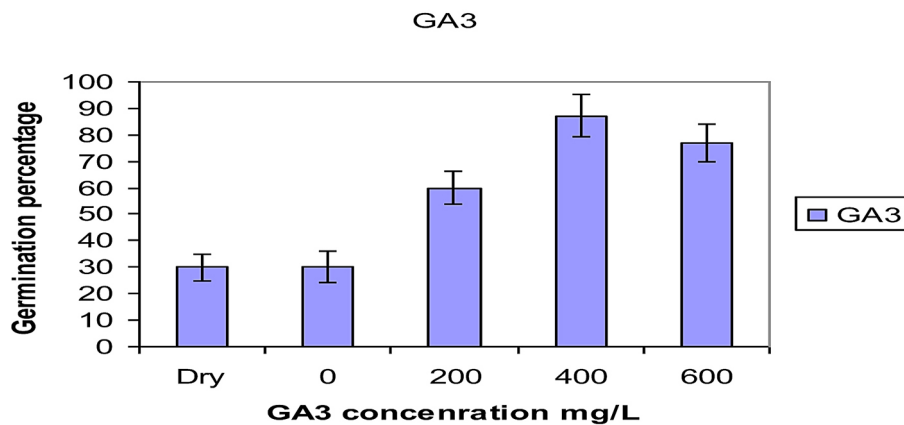


Figure 1. Germination percentage of *G. tournefortii* seeds as influenced by the concentration of GA₃ (Greenhouse experiment). Bars on the column represent standard error

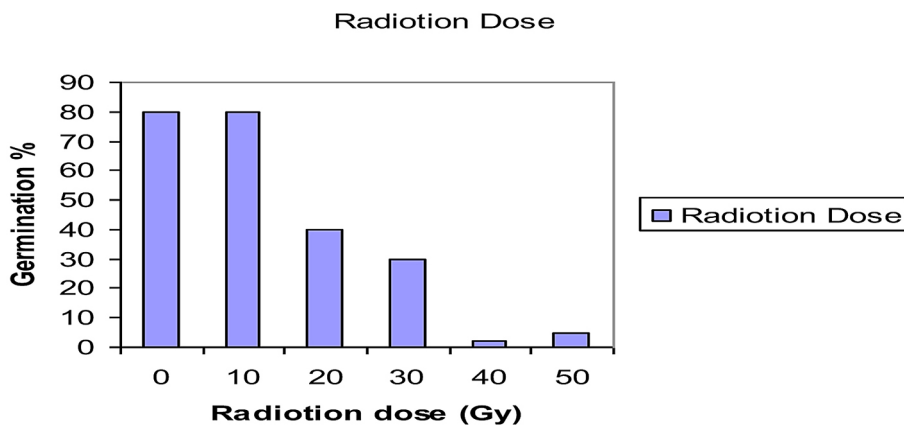


Figure 2. The effect of radiation dose on germination percentage of *G. tournefortii* after treated with 200 mg/L GA₃ for 12 hours. Values represent means of 30 replicates and error bars indicate standard error. Data was collected after 30 days

Impact of gamma radiation

Gamma radiation had a significant influence on seed germination (Figure 2). After 30 days, there was a significant difference in seed germination, increased dose decreased seed germination (Fig. 2). Germination rate was reduced after the radiation treatment in treatments of 40 or 50 Gy and following 28 days in culture. 10 or 20 Gy had no effect on germination percentage. When the seeds were treated with different irradiation doses, the viability of the seeds decreased significantly with radiation dose. The majority of seeds in the highest radiation treatment (46.3 Gy) had a significantly reduced survival rate compared with the control (Figure 2).

DISCUSSION

Shoot culture initiation

The primary goal of this project was to evaluate the seed germination percentage, induction of mutation and to develop effectively *in vitro* propagation for *G. tournefortii* medicinal plant. Contamination is a major problem to overcome when initiating explants, especially when using plant material obtained from the field (Shatnawi, 2013; Al-Ajlouni et al., 2015; Alrayes et al., 2018). Certain decontaminants can cause tissue death if the application period is prolonged. Therefore, the choice of decontaminant is important and the time of application should be optimized, in such way that the treatment is effective in removing contaminants while causing minimum injury to the tissue (George and Sherrington, 1984; Shatnawi et al., 2011; Alrayes et al., 2018). Thus, these results may be explained by explant physiology and endogenous levels of regulators. Moreover, it may be due to the balance between the exogenous growth (George and Sherrington, 1984; Shatnawi et al., 2011; Shatnawi et al., 2013).

After four weeks of observation, explants gave healthy cultures; those healthy cultures were maintained and continually subculture to obtained good culture. The addition of BAP or kinetin to the media was required for shoot multiplication. Supplement of BAP and kinetin improved the development of new shoots/explants (Table 1). Previous studies showed that plant hormones play an important role in microshoots propagation (George and Sherrington, 1984; Shatnawi, 2013; Al-Ajlouni et al., 2015). Of the two cytokinins

tried, BAP produced a maximum number of shoots, compared with kinetin. This may as a result of the elimination of apical dominance that led to the development of multiple shoots and decreased shoot length (Table 1). A similar result was found by *Stevia rebaudiana* (Shatnawi et al., 2011). BAP was also used to induce multiple shoots in *Ruta graveolens* (Al-Mahmood et al., 2012) and *Stevia rebaudian* (Debnath, 2008).

In vitro root formation

In the current study, shoots were able to root without auxin treatment. Root initiation was attained at the bottoms of the shoots, with the existence of IBA, NAA, and IAA. This is similar to the previous results by Shatnawi et al. (2019) of *Ficus carica* (Shatnawi et al., 2015). Callus formed at the shoot bases of *G. tournefortii*, in the presence of IBA, NAA and IAA. NAA, and IBA showed to be the most commonly used growth regulators used for root induction (Table 2). IBA demonstrates enhancing effect for *in vitro* root formation. Moreover, NAA was prompting rooting, and the root was quite the same. This is the first report on the clonal propagation of *G. tournefortii*. The processes defined are highly duplicable and capable. These can be utilized for the clone production plant. This is a simple, economical, rapid, and highly reproducible method to obtain more plantlets within a short time period.

Seed Germination

The viability percentage of *G. tournefortii* seeds after treatment with 1% solution of 2, 3, 4-triphenyl tetrazolium chloride (TTC) was 90%. This indicates that seeds could be capable of germination under favorable conditions and that failure of germination would not be attributed to non-viability of the seeds, but mainly to seed dormancy. The high viability percentage resulted from the fact that these seeds were fresh. The tetrazolium chloride method has gained popularity mainly because of its simplicity, ease, and rapidity of application (França-Neto and Francisco-Carlos, 2019).

Seed is the central way of multiplication of many plants (Lee et al., 2003; Urbanova and Leubner-Metzger, 2018). A highly significant effect of the GA₃ concentration on seed germination was observed using 100, 200 and 300 ppm GA₃ which resulted in the highest germination

percentage (80%). Using a high level (300 ppm) of GA₃ reduced seed germination (Fig 1). A highly significant effect of the GA₃ concentrations on the *G. tournefortii* germination is shown in Figure (1) indicating significant differences between dry seeds and those soaked in water 30%. A significant effect of soaking duration of the *G. tournefortii* seeds in GA₃ on germination was detected (Fig. 1).

Germination percentage increased drastically (up to 90%) when soaking with GA₃ for 12 h. This experiment represented a germination protocol for *G. tournefortii*. Seeds are considered good starting material for *in vitro* culture establishment due to their high viability and germination percentage (Idu et al., 2007; Urbanova and Leubner-Metzger, 2018). Gamma radiation had a significant effect on seed germination. After 30 days, there was a significant difference in seed germination, where increased dose decreased seed germination. The effect of gibberellic acid (GA₃) in improving seed germination was confirmed in many plants. GA₃ has many uses in the agriculture and horticulture industry because of its positive effect on plant development and growth (Idu et al., 2007; Urbanova and Leubner-Metzger, 2017).

Gamma radiation

Gamma radiation had a significant effect on seed germination. The germination rate was reduced after radiation treatment in treatments of 40 or Gy and following culturing in a soil mixture with these treatments. In contrast, 10 or 20 Gy had no effect on germination rate. However, not all seeds exposed to radiation treatment had germination different from the controls ($p > 0.05$). The majority of seeds with high radiation treatment (46.3 Gy) had a significantly reduced survival rate (Fig. 2). Perhaps the most interesting mutant was found in the highest gamma irradiation treatment, which decreased the germination rate (Al-Safadi et al., 2000).

CONCLUSIONS

In conclusion, the results obtained in this study could be useful for the mass propagation of *G. tournefortii* endangered plant. This protocol suggests quick reproduction of plants under *in vitro* controlled environments all year round.

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