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OPTIMIZATION OF MICROPROPAGATION PROTOCOL AND SECONDARY METABOLITES OF *GARDENIA JASMINOIDES* PLANT

Iman M. El-Sayed[a*], Rasha A. El-Ziat[b], Soha A. Murad[c], Lobna S. Taha[a] and Mona H. Mahgoub[a]

[a*]Department of Ornamental Plants and Woody Trees, Agricultural and Biological Research Division, National Research Centre (NRC), Egypt.
[b]Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
[c]Department of Plant Biochemistry, National Research Centre, 33 El Bohouth St. (former El Tahrirst.), Dokki, Giza, Egypt.

Abstract

The present work was carried out to study the effect of different factors such as the type of media and BA at different levels (0.5, 1.0, and 2.0mg/l) on *in vitro* culture establishment. The influence of the physical state of culture media (solid, semisolid and liquid) and NAA at rates of (0.0, 0.2, 0.4, and 0.6mg/l) on rooting behavior was also studied. Secondary metabolism was studied as well to obtain a suitable protocol for micropropagation of the *Gardenia jasminoides* plant and secondary metabolites. For culture establishment, the results showed that adding 2.0mg/l of BA to WPM gave the highest number of shootlets/ explant and leaves/shootlet as compared to control and other treatments while B5 medium supplemented with BA at 1.0mg/ l formed the highest shoot length. The highest rooting rate (100%) was obtained on a solid medium containing NAA 0.4mg/l. The maximum number of roots/shootlet and the longest roots were found on the semisolid medium with any concentration of NAA. The highest content of total chlorophyll and carotenoids were recorded for MS medium supplemented with 2.0mg/ l BA. The best medium for increasing flavonoids was WPM medium enhanced with 2.0mg/l BA, while the superlative culture medium used for increasing phenol compound was MS medium enhanced with 0.5mg/l BA. The highest values of antioxidant in shootlets were shown on B5 medium supplemented with BA at 2.0mg/l followed by WPM medium supplement with BA at 0.5mg/l, compared with vitamin c and all treatments.

Key words: *Gardenia Jasminoides*, *in vitro,* BA, NAA, physical state.

Introduction

Gardenia plant (*Gardenia jasminoids*) belongs to Rubiceae family. There are more than 2 hundred Gardenia species, two out of which constitute the bulk of species of gardenia (*G.thunbergia* and *G.jasminoides*) used. Gardenia is usually used as borders or ground covers and hedges. In order to propagate Gardenia, cutting or grafting may be used. The common uses of *G. jasminoides* fruit include treatment of inflammation, edema, fever, headache, hypertension and hepatic disorders, as was reported (Koo *et al*., 2006).

The growth of plant tissue or organ in aseptic culture on various concentrations of nutrients and hormones that are completely controlled is named plant tissue culture (Razdan, 2003). In tissue culture, rooting of shootlets has been completed using media enriched with auxins both solely or in combination with cytokinin, such as a mixture of indole butyric acid and kinetin (Nazir *et al*., 2011; Venkatachalam and Jayabalan, 1997).

The micropropagation tool success is influenced by many factors such as plant growth regulators, media, and genotype (Gomes and Canhoto 2003; Lin *et al*., 2000; Pati *et al*., 2005) The culture media that were used to enhance shoot induction include commonly used culture media, Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), Woody plant medium (WPM) (Lloyd and McCown,1980) and Gamborg's medium (B5) (Gamborg *et al*., 1968).

The objective of this research is to study the influence of different factors, such as the type of media and BA concentrations, on *in vitro* shooting behavior and the physical state of culture media and NAA concentrations on *in vitro* rooting behavior to optimize a suitable protocol

Author for correspondence : E-mail: imanelsayed6065@yahoo.com

for micropropagation as well as secondary metabolites of the *Gardenia jasminoides* plant.

Materials and Methods

This research was carried out during two years (2018 and 2019) on *Gardenia jasminoides* at Central laboratories, Tissue Culture Technique laboratory, Dep. of Ornamental Plants and Woody Trees and Dep. of Plant Biochemistry, National Research Centre (NRC), Egypt.

Plant material and surface sterilization

Plants (2 years old) of Gardenia were collected from the nursery of Central laboratories, Department of Ornamental Plants and Woody Trees, National Research Centre (NRC). The explants (stem node) were rinsed then sterilized in ethyl alcohol 70% (v/v) for one minute, sodium hypochlorite 15% (Clorox) for ten minutes, then $HgCl_2$ 1% for ten minutes and finally washed 3 times with sterile distilled water.

Culture medium

For the shootlet multiplication stage, three different culture media MS, WPM (Lloyd and McCown, 1980) and B5 (Gamborg *et al*., 1968) were used and were added with various levels of benzyladenine (BA) (0.5, 1.0 and 2.0mg/l). The media were enriched with 25g/l sucrose, pH at 5.7±1 and agar, as a solidifying agent, at 7g/l. The following data were detected as shootlet number per explant, shoot length (mm) per explant and leaves number formed per shootlet.

Three physical states (solid, semisolid, and liquid) of MS culture media were augmented with various levels of 1-Naphthalene acetic acid (NAA) (0.0, 0.2, 0.4, and 0.6mg/l) for *in vitro* rooting stage that was determined after 8 weeks from the culture of shootlets. The following data were tabulated as a percentage of roots (%), roots number per shootlet and roots length (mm).

Culture incubation

All stages of explants were cultured in incubation conditions at 25± 2 ºC under controlled white light of cool fluorescent tubes with an intensity of lighting 3000 lux day light above cultures; the photoperiod was 8 to16 (day per night) organized by an electronic timer.

Hardening off

The obtained plantlets were transplanted to plastic pots holding a different mixture of soil (sand to peat moss to perlites 1:1:1 v/v/v) and covered with plastic bags for about 1 month and the percentage of surviving plants (%) was recorded.

Photosynthetic pigments

One gram fresh weight of shootlets tissues was used for the determination of photosynthetic pigments (total chlorophyll), as well as carotenoids as mg/g fresh weight, using spectrophotometer, according to the procedure achieved by (Saric *et al*., 1967).

Secondary metabolites estimation

Preparation of extraction

Ten mg of fresh leaves were extracted with 5ml acetone (85%), then filtered and extracted twice. Phenolic compound and antioxidant capacity were determined in this final extraction.

Total phenol and flavonoids

The phenols content was determined using Folin- Ciocalteu reagent method (Singleton *et al*., 1999) and flavonoids content was assayed using the Folin - Ciocalteu method (Tambe and Bhambar, 2014).

Total antioxidant capacity

The total antioxidant activity assay was carried out (Prieto *et al*., 1999) according to the phosphomolybdenum method.

Protein content %

The soluble protein content was carried out using coomassie brilliant blue G-250, as a protein binding dye at 595 nm, according to Bradford (1976).

Statistical analysis

The data were obtained using a randomized complete block design with three replicates of each treatment. The treatments means were compared for significance by Duncan's New Multiple Range test at 0.05% level of probability (Duncan, 1955) using COSTATV-63.

Results and Discussion

In vitro shoot behavior

The influence of various culture media (MS, WPM and B5) augmented with different concentrations of BA (0.5, 1.0,and 2.0 mg/l) on the formed shootlets number/ explant, length of shootlets and the number of leaves formed per shootlet were recorded in table 1. The data indicated that adding BA at 2mg/l to WPM culture medium gave the largest number of shootlets/explant (7.3), compared with control (MS free of hormones) and other treatments, while adding BA at 1mg supplemented to B5 produced the longest shoot (48.1mm) followed by BA at 2 mg/l supplemented to B5 medium. WPM culture medium augmented with BA at the rate of 2 mg/l resulted in the greatest number of leaves/shootlet (56.6), whereas

Table 1: *In vitro* shooting behavior affected by culture media types and BA concentrations.

Characters / Treatments	No. of Shootet/ explant	Shoot length (mm)	Leaves number/ shootet
Control (MS free hormones)	3.4 ^{e}	27.2 ^{cd}	37.0 ^{cd}
MS +BA 0.5mg/l	5.5 ^{cd}	21.4 ^{d}	51.0 ^{b}
MS+BA 1mg/l	6.5 $^{a-c}$	22.6 ^{d}	47.6 ^{b}
MS+BA 2mg/l	6.9 ^{ab}	26.0 ^{cd}	51.3 ^{b}
WPM+BA 0.5mg/l	6.1 $^{b-d}$	35.0 ^{b}	31.6 ^{e}
WPM+BA 1mg/l	6.1 $^{b-d}$	31.4 ^{bc}	41.3 ^{c}
WPM+BA 2mg/l	7.3 ^{a}	25.5 ^{cd}	56.6 ^{a}
B5+BA 0.5mg/l	5.4 ^{d}	34.4 ^{b}	37.8 ^{cd}
B5+BA 1mg/l	6.4 $^{a-d}$	48.1 ^{a}	48.1 ^{b}
B5+BA 2mg/l	6.5 $^{a-c}$	35.2 ^{b}	35.2 ^{d}

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range tests at 5% level of probability.

MS= Murashige and Skoog medium, BA= Benzyladenine, WPM=Woody plant medium, B5= Gamborg's medium.

untreated treatment (MS culture medium free hormones) gave the lowest values of number of shootlets, shoot length and leaves numbers (3.4, 27.2 mm and 37.0, respectively). The lowest level of BA at (0.5mg/l), which was added to B5 or WPM culture media, seemed to have a significant promotion effect on shootlets elongation (34.4 and 35.06 mm, respectively).

These results are in agreement with those obtained by Khaleghi *et al*., (2008) on *Alstroemeria cv.* Fuego. They found that the highest number of shoots was found on the medium enhanced with 1.5 mg/l BA and 0.2 mg/l NAA.

Auxin and cytokinin are the main phytohormones that are effective for plant growth and development involved in cell division, growth and organ differentiation (Hu and Wang, 1983). The plant growth regulators dose in the culture medium affects the growth of shoots or roots formation (Joshi *et al*., 2009).

For Gardenia micropropagation, MS culture medium supplemented with vitamins was used as well. WPM was successfully used for micropropagation of recalcitrant woody species (Lloyd and McCown, 1980). This might be attributed to the fact that the multiplication of shoots could be formed on WPM containing a lower concentration of nitrogen and potassium than those of MS medium (Cervera *et al*., 2008; Kobayashi *et al*., 2003)

In vitro rooting behavior

The tabulated data in table 2 show that the rooting behavior of *Gardenia jasminoides* shootlets was affected

Table 2: *In vitro* rooting behavior affected by culture medium systems and NAA concentrations.

Characters / Treatments	Rooting %	No. of Roots/ shootlet	Root lenght (mm)
Control (MS free hormones)	63.8 ^{b}	2.6 ^{d}	25.7 ^{e}
Soild MS +NAA 0.2mg/l	91.6 ^{a}	11.3 ^{a}	24.3 ^{e}
Soild MS +NAA 0.4mg/l	100 ^{a}	11.0 ^{a}	34.8 ^{d}
Soild MS +NAA 0.6mg/l	91.6 ^{a}	9.6 ^{ab}	39.3 ^{cd}
Semi-soild +NAA 0.2mg/l	100 ^{a}	5.0 ^{cd}	48.8 ^{b}
Semi- soild +NAA 0.4mg/l	100 ^{a}	6.0 ^{cd}	63.0 ^{a}
Semi- soild +NAA 0.6mg/l	100 ^{a}	7.0 ^{bc}	44.1 ^{b}
Liquid+NAA 0.2mg/l	58.3 b	6.0 ^{cd}	37.6 ^{cd}
Liquid+NAA 0.4mg/l	58.3 b	5.3 ^{cd}	24.3 ^{e}
Liquid+NAA 0.6mg/l	66.6 b	5.0 ^{cd}	10.5 ^{f}

Means having the same letter(s) within the same column are not significantly different according to Duncan's multiple range tests at 5% level of probability.

MS= Murashige and Skoog medium, NAA= 1-Naphthalene acetic acid.

by various culture media states (solid, semisolid and liquid culture medium) supplemented with various levels of NAA (0.0, 0.2, 0.4 and 0.6 mg/l). The results indicate that the highest percentage of rooting (100%) was recorded on the solid medium with NAA 0.4mg/l and semisolid with all used concentrations of NAA, while the lowest

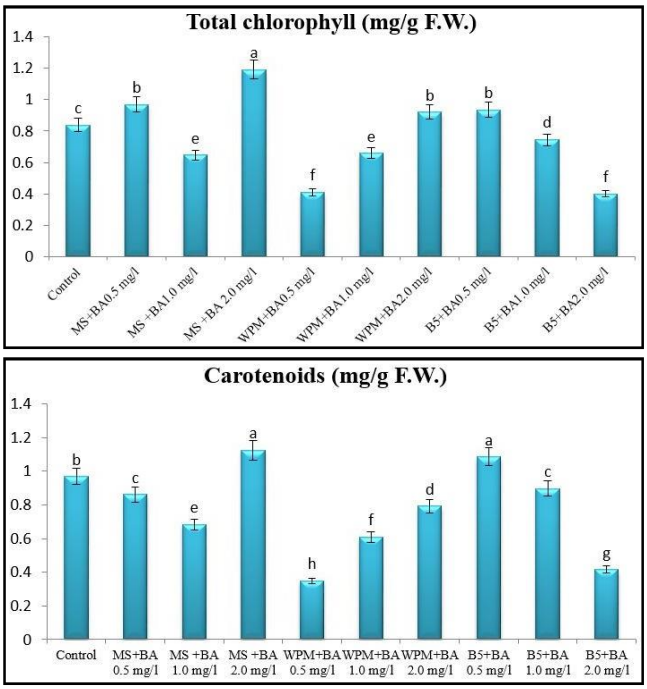

Fig. 1: Pigments content (mg/gFW.) *in vitro* propagated *Gardenia jasminoides* as affected by different culture media and BA concentrations.

MS= Murashige and Skoog medium, BA= Benzyladenine, WPM = Woody plant medium, B5= Gamborg's medium.

percentage of rooting was found on liquid medium augmented with NAA at 0.2 and 0.4 mg/l (58.33 and 58.33%). However, the greatest number of roots/shootlet was recorded in the solid medium supplemented with any level of NAA gave (11.33, 11.0, and 9.67), while the lowest number of roots (2.67) was recorded for control (free hormone). The root length on semisolid medium added with any concentration of NAA (0.2, 0.4, and 0.6 mg/l) gave the longest roots (48.8, 63 and 44.13mm), compared with control and other treatments used.

These results are in agreement with Rout *et al.*, (1999) who suggested that the rooting of microshoots was best on solid medium compared with other media. Seyyedyousfi *et al*., (2013) on Alstroemeria found that using MS culture medium with 1.0 mg/l NAA gave the highest root and a maximum number of roots. It seemed

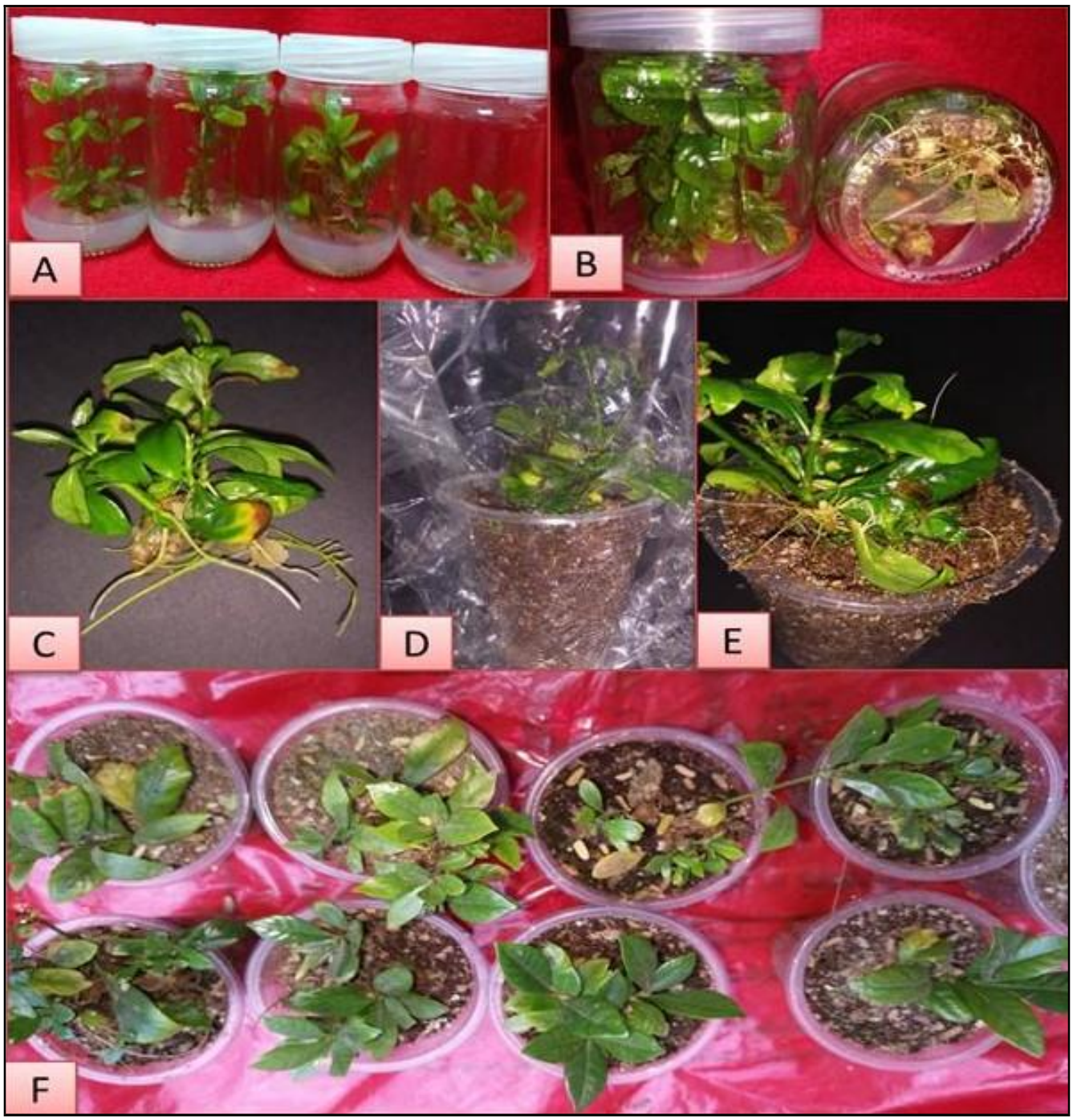

Fig. 2: Shooting and rooting induction of *Gardenia jasminoides on in vitro* culture (A): Shootlets improvement that was formed from using WPM plus 2mg/l of BA, (B): Rooting shootlets cultured on the same solid plus NAA at 0.2, 0.4 and 0.6 mg/ l (C, D) : Acclimatized plants to greenhouse.

that NAA was able to stimulate root induction that was an effective growth regulator for root formation (Lin *et al*., 2000).

Effect of soil mixtures on hardening off *Gardenia jasminoides* plantlets

Rooted plantlets of *Gardenia jasminoides* that were obtained from *in vitro* culture on semi-solid or solid MS culture media were successfully acclimatized (90-100 % survival)when they were transferred to the greenhouse in the sand: peat moss: perlite (1:1:1 v/v/v).

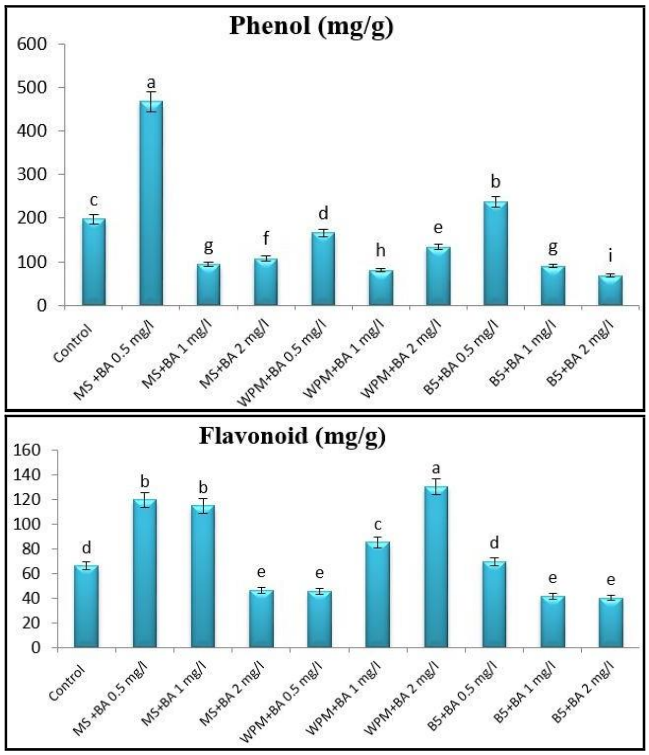

Fig. 3: Effect of culture media and BA concentrations on secondary metabolities compounds *in vitro* of *Gardenia jasminoides.*

MS = Murashige and Skoog medium, BA = Benzyladenine, WPM = Woody plant medium, B5 = Gamborg's medium.

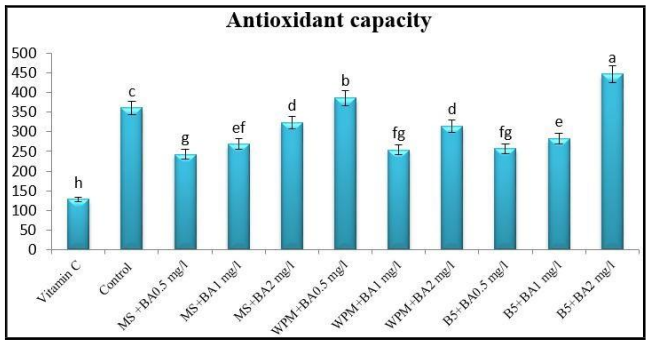

Fig. 4: Effect of culture media and BA concentrations on antioxidant capacity *in vitro* of *Gardenia jasminoides.*

MS = Murashige and Skoog medium, BA = Benzyladenine, WPM = Woody plant medium, B5 = Gamborg's medium.

Pigments content of *Gardenia jasminoides* shootlets affected by different culture media

The effect of different culture media (MS, WPM and B5) added with BA levels (0.0, 0.5, 1.0 and 2.0mg/l) also influenced the chlorophyll content of *Gardenia jasminoides* in Fig. 1. The data presented show that total chlorophyll and carotenoids took the same trend. The highest values of total chlorophyll and carotenoids content were recorded with MS medium supplemented with BA 2.0mg/l (1.193 and 1.125mg/g F.W., respectively). Also, Shootlets that were obtained from B5 supplemented with BA at 2.0mg/L contained total chlorophyll within the lowest values (0.399 mg/g FW.). The lowest carotenoids content (0.348 mg/g FW.) in shootlets was obtained from WPM supplemented with BA 0.5mg /l.

These results are in agreement with Abou-Dahab (2005) who reported that the strength of MS medium gave the greatest chlorophyll contents of *Ruscus hypoglossum*, Nur Inani.

Fakhrul *et al.*, (2014) stated that macronutrients manipulation in MS medium could have an effect on the growth and chlorophyll in *Stevia rebaudiana*.

Effect of culture media and BA concentrations on secondary metabolites compounds *in vitro*

Data in Fig. 3 show that the highest contents of flavonoids was recorded in shootlets that were *in vitro* on WPM culture medium with BA at 2.0mg/ l (130.55 mg/g). On the other hand, B5 supplemented with BA 2.0mg/l resulted in the reduction of the flavonoid (40.22 mg/g.), while phenol compounds in shootlets obtained from MS supplemented with BA at 0.5mg/l recorded the highest value (467.33 mg/g), compared with control and other treatments. Meanwhile, the lowest

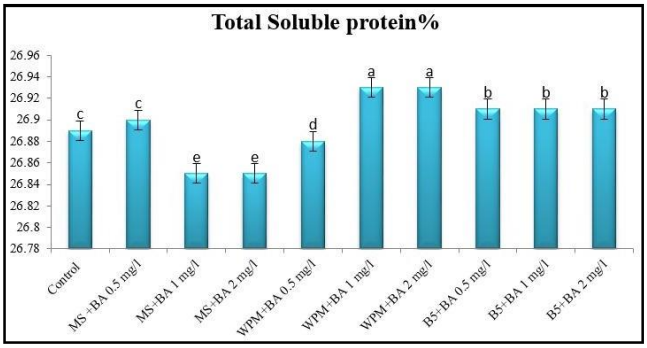

Fig. 5: Effect of culture media and BA concentrations on total soluble protein%. *in vitro* of *Gardenia jasminoides.*

MS = Murashige and Skoog medium, BA = Benzyladenine, WPM = Woody plant medium, B5 = Gamborg's medium.

value of phenols was recorded on B5 supplemented with BA 2.0 mg/l (68.19mg/g.).

These results are in agreement with those obtained from Paric *et al*., (2017) on *Mentha piperita* explants that were cultured *in vitro.* They mentioned that using the highest rate of BA affects the production of phenolic compounds.

Earlier reports suggested that the production of secondary metabolites stimulates multiplication and division subsequently. The major role of plant growth regulators is bound to determine and obtain culture potentiality. The existence of BA in the culture medium promotes proliferation of shoots and flavonoid content (Pasqua *et al*., 2003).

Effect of culture media and BA concentrations on antioxidant capacity *in vitro* of *Gardenia jasminoides*

The data in Fig. 4 indicate that antioxidant capacity in shootlets was in the highest value (446.83) in the shootlets cultured on B5 medium with BA at 2mg/l followed by that was in shootlets cultured on WPM medium with the addition of BA 0.5mg/l (386.51) and then MS medium supplemented with BA at 2mg/l (323.41) compared with all treatments. The lowest value was observed in culture on MS medium augmented with BA0.5mg/l (242.46). These results were confirmed by those obtained by Paric *et al*., (2017) on *Mentha piperita* explants cultured *in vitro*. They mentioned that the production of antioxidant activities was affected by several BAP and IBA treatments.

Growth and secondary metabolite biosynthesis in plant micropropagation is crucially influenced by plant growth regulators (Qian *et al*., 2009). The auxin to cytokinin ratio seemed to be the main factor to control growth and morphological characters (Sharafzadeh and Zare, 2001). Plant growth regulators applications have a significant effect on the metabolism of secondary metabolites (Khan *et al*., 2008).

Protein content %

Soluble protein with Bradford was estimated in shootlets that were obtained on all culture media used (MS, WPM and B5) and supplemented with 0.5, 1 and 2mg/l of BA. As it is shown from the presented data, it was found that BA at all concentrations used had no significant effect on the content of protein in the *Gardenia jasminoides* plant. This content ranged from 26.83-26.93% Fig. 5.

Conclusion

We can conclude that the highest significant value of the number of shootlets/explant and the number of leaves/shootlet resulted from adding 2.0mg/l of BA to WPM. The highest percentage of rooting (100%) was recorded on the solid medium with NAA 0.4mg/l, while the semisolid medium with all used concentrations of NAA gave the maximum number of roots/shootlet and the longest roots. MS medium supplemented with 2.0mg /l BA gave the highest content of total chlorophyll and carotenoids. WPM medium supplemented with 2.0 mg/l BA was the best medium for increasing flavonoids, while MS medium supplemented with 0.5mg/ l BA was the superlative culture medium used for increasing phenol compound. Antioxidant capacity in shootlets had the highest values on the B5 medium supplemented with BA at 2.0mg/l compared with all treatments.

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