



Polyamine Elicitation of Quercetin and Rutin Production in Callus Cultures of Caper and Impact to Regeneration

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Nowadays, efforts to produce high amounts of secondary metabolites with anticancer properties in plants have become remarkable studies of biotechnological approaches. Polyamines are known to have successful roles in plant metabolism and molecular signalling. The important point is to determine the type and concentration of polyamine suitable for the physiology of the plant. This study was carried out to examine the effects of different polyamine applications in callus culture of Caper (*Capparis L.*) plant on plant regeneration and the production of quercetin and rutin with anticancer properties. For this purpose, different varieties (spermine, spermidine, and putrescine) and concentrations (0.0, 0.5, and 1.0 mg/l) of polyamines and 2.0 mg/l 6-benzylaminopurine (BAP) and 1.0 mg/l α -naphthaleneacetic acid (NAA) of plant growth regulators were added to the Murashige and Skoog (MS) culture medium. According to the results, the highest value in terms of callus induction was determined in 1.0 mg/l spermine, the highest value in plant regeneration was obtained in 0.5 mg/l spermine, and the highest quercetin and rutin accumulation were detected in 0.5 mg/l spermidine application. The regeneration and the amount of quercetin increased approximately 2-fold compared to the control group in the aforementioned polyamine (PA) applications. Our findings showed that spermine and spermidine proved more effective in the callus culture of Caper than putrescine, and the use of low-dose polyamines gave outstanding results. Therefore a particularly valuable finding obtained in this study is that polyamines can be used as an effective elicitor in the tissue culture of Caper plant.

Keywords: Callus induction, Caper, Quercetin, Regeneration, Rutin, Secondary metabolites

Introduction

Due to the increasing demand for herbal products and natural medicines, studies on tissue culture with medicinal and aromatic plants have become the point of focus. Currently, the use of various elicitors in callus culture is an important tool for the cultivation of medicinal plants, stable quality, and production of valuable secondary metabolites that are not adequately supplied.¹ The important point is to maximize the production level by using a suitable type-specific elicitor. Polyamines (PAs) are low molecular weight amines in aliphatic structure that are involved in developmental and physiological events such as cell differentiation and division,² promote totipotency and help molecular signalling.³ The external application of PAs has been used by many researchers to increase or initiate cell division and growth.⁴ The most common types of PAs are spermine (SPM), spermidine (SPD), and their diamine precursor putrescine (PUT). In plants, both the biosynthesis and mechanisms of action of PAs have been less studied than in other organisms.

Plant species belonging to the Capparidaceae family, known as Capers, are common mainly in warm regions of the world, with *Capparis spinosa* L. and *Capparis ovata* Desf. being the most widely studied species. The Caper plant, known for its therapeutic as well as nutritional purpose, is used in the treatment of cancer diseases with its anticarcinogenic,⁵ antirheumatic, antibacterial,⁶ antifungal, antiallergic, anti-inflammatory, antioxidant effect and contains pharmacologically interesting secondary plant metabolites as they may play a role in reducing the risk of tumor formation.⁷ The leaves and buds of the Caper plant are very rich in phenolic compounds.^{8,9} *C. spinosa* L. contains significant amounts of quercetin and rutin. Quercetin, which is one of the bioactive molecules, is one of the important compounds of Capers with a very rich source of antioxidants.¹⁰ In addition to its anticarcinogenic effect, rutin, also known as vitamin P, which prevents the deterioration of the vascular structure, is one of the other valuable phenolic compounds of Caper.¹¹

It has been observed that many studies on the Caper plant have been carried out on the purpose of its propagation in order to meet the increasing demand for its seedlings. This is because of the low germination

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potentials of its seeds.¹² Tissue culture techniques offer the possibility to generate varieties of secondary metabolites using different synthesis pathways of plant cells.¹³ Callus and suspension cultures are capable of totipotency for the biosynthesis of secondary metabolites, as they contain the complete genetic information of a whole plant. In this case, consistently high quality secondary metabolites can be produced. There has not been any study on the effects of PAs on the amount of quercetin and rutin, and on plant regeneration in the callus culture of Caper plant. This study was aimed to investigate the effects of PAs (SPM, SPD and PUT) on callus induction, plant regeneration, quercetin and rutin quantity, and determine the most effective quantity and type of PA.

Material and Methods

Plant Material

Seedlings of *Capparis* L. was used as donor plants. Plants were grown in a plant growth room under controlled conditions on a photoperiod of 16/8 h light/dark, the temperature at $24 \pm 2^\circ\text{C}$; 18 ± 2 h light, 12 000 lux light intensity, and relative humidity of 51–54% (PeakTech 3695) in Biotechnology Laboratory in the Biology Department of Suleyman Demirel University.

Sterilization

Caper leaves were used as explants for the initiation of culture. The leaf explants collected from the plant were washed thoroughly under running tap water to get rid of soil particles. Explants were first immersed in 70% (v/v) ethanol for 10 min and then rinsed with autoclaved distilled water three times (1 min each). Then, explants were kept for 10 min in a 15% (v/v) solution of NaOCl plus two drops of Tween-20 and finally washed with sterile distilled water three times (1 min each).

Callus Induction and Maintenance

For the purpose of callus initiation sterilized explants of Caper were inoculated on the surface of solid MS medium (Sigma M5519) supplemented with plant growth regulators of 2.0 mg/l BAP and 1.0 mg/l NAA. Different types (SPM, SPD and PUT) and concentrations (0.0, 0.5 and 1.0 mg/l) of PAs (Khalil *et al.* 2016) were added to the nutrient medium to observe the differences in callus induction. After the addition of 7 g/l agar and 30 g/l sucrose, the pH of the medium was adjusted to 5.8 with (0.1 and/or 1 N) NaOH and/or (0.1 and/or 1 M) HCl. Then, medium autoclaved at temperature of 121°C and 1.1 atm pressure (1 kg/cm^2) for 20 min.

Sterilized explants were dissected into 0.5–1 cm discs with a sterile scalpel and cultured on MS media with an average of 16 leaf discs per petri dish of 90 mm diameter. The explants were incubated in the dark at $25 \pm 2^\circ\text{C}$. Callus induction was observed from leaf explants at the end of approximately 40–45 days. Before the calli were taken into the regeneration medium, callus induction rates, diameters and weights were measured, as well as their colors and types were also determined.

Callus induction rate (%) =

$$\frac{\text{Number of callus forming explants}}{\text{Total number of explants in petri dish}} \times 100$$

Measurement of Callus Weights: The weight (g) of each callus was measured with the help of precision scales in a sterile cabinet.

Measurement of Callus Diameters: Measurements (cm) of each callus were made with the help of a ruler in a sterile cabinet.

Callus colour: white, yellow, greenish, or brown colors of the calli were determined visually.

Callus texture: compact or friable structure of the calli was determined visually.

Regeneration

MS (Sigma M5519) culture medium was used to promote shoot and root formation from calli obtained from explants. The medium was supplemented with plant growth regulators 0.4 mg/l BAP and 1.0 mg/l NAA with the addition of 7 g/l agar and 30 g/l sucrose. Different types (SPM, SPD and PUT) and concentrations (0.0, 0.5 and 1.0 mg/l) of PAs were added to the MS medium and the pH of the medium was adjusted to 5.8 with (0.1 and/or 1 N) NaOH and/or (0.1 and/or 1 M) HCl. Afterwards, the media autoclaved at a temperature of 121°C and 1.1 atm pressure (1 kg/cm^2) for 20 min.

Callus was induced 40–45 days after the explants were transferred to the medium prepared for regeneration. Calli were regenerated by keeping them in an incubator for 30 days at $25 \pm 2^\circ\text{C}$, with a photoperiod of 16/8 h light/dark.

Determination of Phenolic Compounds

After callus induction, cultures were subcultured twice in a PA-containing MS culture medium. On day 12 of subculturing, when the cells were in the rapid or multiple growth phase, the calli were transferred to sterile falcon tubes and stored at -20°C until the analysis of quercetin and rutin.

In order to determine the content of phenolic compounds, 2 g callus samples crushed with liquid nitrogen were homogenized with a homogenizer with 10 ml of 96% ethanol for 2 min. The mixture was then kept in a water bath at 45°C for 14 hours. Then, the homogenate was centrifuged at $2350 \times g$ -force (RCF) for 5 min. The supernatant containing phenolic compounds was taken and evaporated in a rotary evaporator at 45°C until completely dry. Afterwards, the extracts were dissolved in 500 μ l of methanol. After filtering through a 0.22 μ m Millipore filter, 20 μ l samples were injected into high performance liquid chromatography (HPLC).

HPLC Analysis

Phenolic analysis was performed with HPLC device (Shimadzu) by Suleyman Demirel University Innovative Technology Application and Research Center. The HPLC system was equipped with a pump (LC-10ADvp), auto-sampler (SIL-10AD vp) and column oven (CTO-10Avp). Agilent Eclipse XDB-C18 (250 \times 4.60 mm, 5 μ m) DAD detector was used as the column, and the UV wavelength of the detector was set to 278 nm. The mobile phase consists of A: 3% acetic acid, B: methanol. The flow rate was set at 0.8 ml/min, the column temperature at 30°C, and the injection volume at 20 μ l. The examined phenolic compounds were calculated using a calibration blank prepared with their standard solutions, and the results were given as μ g/g fresh weight (FW).

Statistical Analysis

Experiments were carried out according to randomized complete design and all applications were obtained with at least 3 replications. Statistical analysis was conducted to the analysis of variance (ANOVA) using SPSS (ver:23.0). Means were analyzed and grouped using Duncan's Multiple Range Test. Differences were considered statistically significant at the $p < 0.05$ levels.

Results and Discussion

The use of biotic and abiotic elicitors in plant tissue culture media has emphasized the production of medically valuable phytochemicals and their commercial production in the industry.¹⁴ In this study, the observed parameters for the callus induction experiments were callus induction percentage (%), fresh weight (g), diameter (cm), colour and texture of callus. The appropriate dose and type of elicitors used in the culture medium significantly affect callus growth and, therefore, metabolite production.

Statistical analysis ($p < 0.05$) showed that different types and concentrations of PAs in MS medium decreased the frequency of callus induction except for 1.0 mg/l SPM treatment. The highest percentage of callus induction (87.6%) was obtained in 1.0 mg/l SPM group, followed by 81% in the control group and the lowest percentage was in 0.5 mg/l SPD with 38.4% (Fig. 1). Different opinions have been reported about the effects of PAs on callus induction. Thiruvengadam *et al.*¹⁵ reported in their study that PAs have a positive effect on increasing the number of somatic embryos of *Momordica dioica* in the early stages of embryogenic callus growth. Dewi & Purwoko¹⁶ reported that PUT was more effective than SPM and SPD polyamines on callus induction and plant regeneration obtained from anther culture of rice. Similarly, the studies of *Panax ginseng*¹⁷, *Gossypium hirsutum*¹⁸, *Momordica charantia*¹⁹ and *Daucus carota*²⁰, reported PUT is one of the other PA groups that it was more effective in callus induction rate and regeneration. The result obtained from our research opts for the view that PA treatments that decrease callus production are associated with extensive accumulation of secondary metabolites. This was a positive result desired for our study. Because 0.5 mg/l SPD application, the lowest callus induction application, showed the highest secondary metabolite accumulation of quercetin and rutin.

The morphological changes that occur *in vitro* with callus growth can be largely identified for the accumulation of secondary metabolites.²¹ In our study, PA applications had an increasing effect on the fresh weight of callus (Fig. 2). In 0.5 mg/l PUT application, callus fresh weight increased 2.3-fold compared to the control group, followed by 1.0 mg/l SPD with a 1.7-fold increase and 1.0 mg/l PUT with a 1.5-fold increase. Paul *et al.*¹⁹ found in their *in vitro*

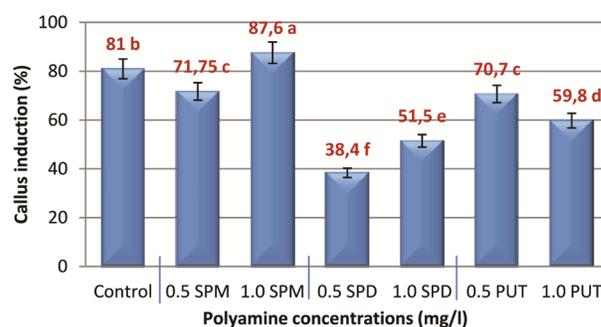


Fig. 1 — Effects of different types and concentrations of PAs on callus induction of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

study of *M. charantia* L. leaf that PAs increased somatic embryogenesis and increased embryogenic callus weight 5-fold, 3.8-fold, and 2.7-fold in 1 mM PUT, 0.1 μ M SPM, and 0.1 μ M SPD applications, respectively. Similarly, Satish *et al.*²² in their study with *Eleusine coracana* plant, the highest number of embryogenic callus and fresh weight increased 1 to 2 fold with 1.5 mM SPD in all the genotypes of *E. coracana* followed by 1.0 and 2.0 mM SPD. According to the results obtained from our study, the highest fresh weight was obtained in 0.5 mg/l PUT application, while the quercetin and rutin ratios decreased compared to the control group. While the highest fresh weight was detected in the application of salicylic acid used among various elicitors in *Phoenix dactylifera*, the decrease in phenolic substances such as agigenin and total flavonoid content is an indication that there may be a negative correlation between fresh weight and secondary metabolite accumulation.²³

When the diameter of the calli obtained from Caper leaf explants was examined, it was observed that the applications of 1.0 mg/l SPM, 0.5 mg/l PUT, 1.0 mg/l PUT and 1.0 mg/l SPD PA had an increasing effect compared to the control group (Fig. 3). PA

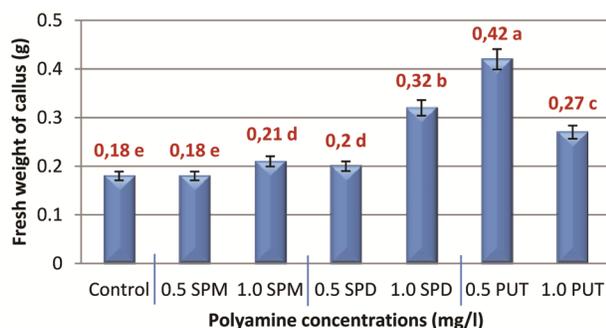


Fig. 2 — The effects of different types and concentrations of PAs on fresh callus weight of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

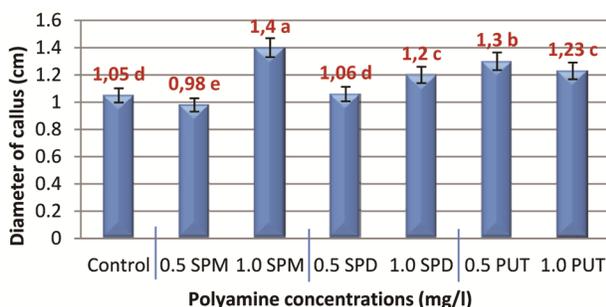


Fig. 3 — Effects of different types and concentrations of PAs on the diameter of callus of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

application with the highest callus diameter was detected in the SPM group with 1.4 cm and 1.0 mg/l ($p < 0.05$). Kumlay and Ercisli²⁴ concluded that the callus induction percentage, diameter and weight of callus, texture, color and degree of callus induction varied according to the different combination of plant growth regulators (PGRs) (BAP; 3.0 mg/l and NAA ; 2.0 mg/l) and the degree of influence between them and explant sources of potato cultivars. In our study, while the largest callus sizes were seen in 1.0 mg/l SPM and 0.5 mg/l PUT applications, quercetin and rutin contents decreased compared to the control group at these doses. The highest determination of callus sizes in these treatments may indicate that PA alters some physiological processes at these concentrations and varieties, such as the phytochemical synthesis process.

It has been determined that the rate of callus induction differs according to the PA concentration and type, but the callus structure was compact in all applications, so PAs did not have a significant effect on the callus structure. The impact of PA applications on callus color was determined as white at 0.5 mg/l SPM and brown at 1.0 mg/l SPM (Fig. 4). Brown occurring in callus cultures is an important morphological character in determining the success of phytochemical accumulation in plant tissue culture. In all other PA groups, two colors were determined as brown/white. In the beginning, the colors of calli ranged from colourless to whitish and later turned into light brown. After two weeks of incubation, the colors of calli changed from golden brown to dark brown. In the 1.0 mg/l PUT group, both brown and white callus was observed, but white callus induction was more common. Ehsandar *et al.*²⁵ and Iqbal *et al.*²⁶ suggested that compact and dark green-brown calli of potato cultivars have a good regeneration ability, and could be used for shoot propagation.

PA supply to the culture medium also favoured the efficient regeneration and accumulation of bioactive secondary metabolites. In this study, the addition of PA to the MS medium also had a positively important effect on the percentages of regeneration in Caper leaf explants. All PA types and concentrations increase the regeneration rate statistically ($p < 0.05$). The most effective regeneration rate was 0.5 mg/l SPM treatment with 16.17%, approximately 2 times higher than the control group (7.81%) (Fig. 5). In addition, the 1.0 mg/l PUT (13.51%) and 0.5 mg/l SPD (13.23%) treatments performed well for regeneration

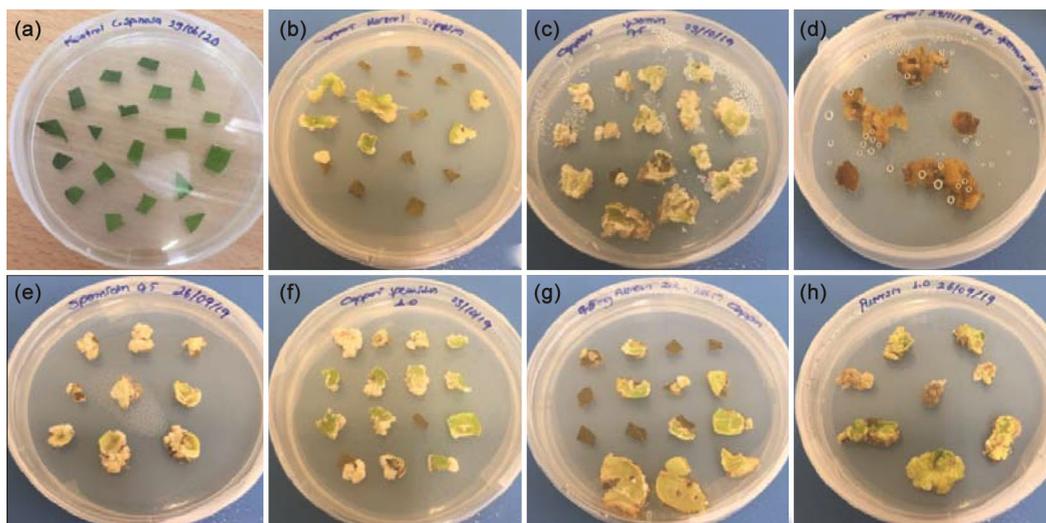


Fig. 4 — Effects of different types and concentrations of PAs on callus formation of Caper leaf explants: (a) leaf discs (b) control, (c) 0.5 mg/l SPM, (d) 1.0 mg/l SPM, (e) 0.5 mg/l SPD, (f) 1.0 mg/l SPD, (g) 0.5 mg/l PUT, (h) 1.0 mg/l PUT

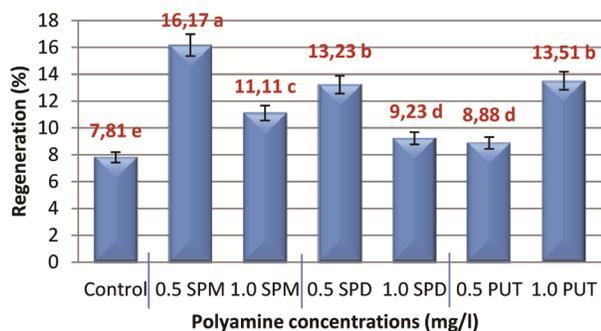


Fig. 5 — The effects of different types and concentrations of PAs on the percentage of regeneration of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

compared to the control group (7.81%). Rubio-rodriguez *et al.*²⁷ stated that SPM with nitrogen deficiency applications gave the best results in secondary metabolite production and *in vitro* regeneration. Diwan and Malpathak²⁸ reported that 80 μ M SPM increased shoot regeneration capacity 2.5 times in *Ruta graveolens* compared to the control group. Additionally, Sakhanokho *et al.*¹⁸ observed that adding PUT to all lines of different media compositions increased the number of somatic embryos and plant regeneration in *Gossypium hirsutum* (upland cotton). Our results reveal that the regeneration rate and the amount of quercetin and rutin increased significantly with 0.5 mg/l SPD application. This indicates the existence of a relationship between *in vitro* regeneration and phenolic compound production.

No information is available on the effect of PA addition in callus culture systems to produce more

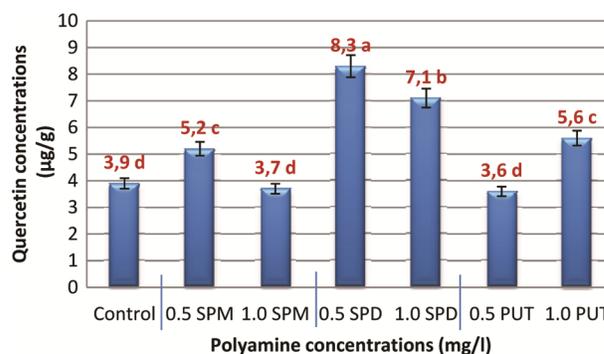


Fig. 6 — The effects of different types and concentrations of PAs on the quercetin content of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

phenolic compounds on Caper species. The effect of PA applications on the amounts of quercetin and rutin was variable. However, SPD applications at both concentrations had a positive effect on the amounts of quercetin and rutin. It was clear from the results that 0.5 and 1.0 mg/l SPD increased the quercetin content (8.3 and 7.1 μ g/g) 2.1-fold and 1.8-fold higher than the control group (3.9 μ g/g) (Fig. 6). Khalil *et al.*²⁹ pointed out that the media supplemented with 2 mg/l 6-benzyleadenine (BA), 2 mg/l kinetin (Kn) and 2 mg/l SPD gave the most stevioside amount when checked against the other combinations of PAs in the growth medium of *Stevia rebaudiana*. Studies have shown that in addition to the positive effects of PAs on the growth and development of plants,³⁰ these substances also have an stimulant effect on the phytochemicals of medicinal plants.³¹ PAs have a direct effect on the production of secondary

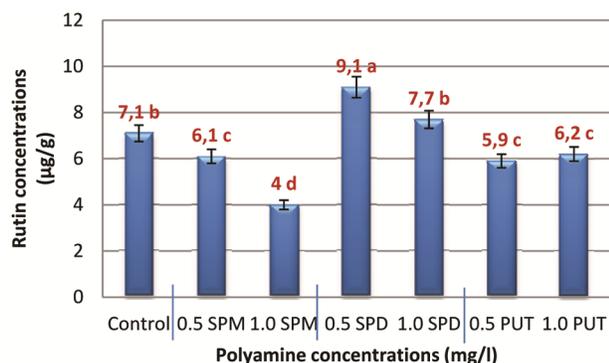


Fig. 7 — The effects of different types and concentrations of PAs on the rutin content of Caper leaf explants; The mean values shown with different letters are significant at the $p < 0.05$ level

metabolites as well as affect the production of metabolites due to plant growth.³² Our results align with previous studies showing that PAs as elicitors increase secondary metabolite content in medicinal plants. Bais *et al.*³³ found that incorporating PUT and SPD into hairy root cultures in the plant *Cichorium intybus* increased betalain and thiophene amounts with increasing biomass accumulation.

SPD treatment also positively affected the rutin content with the addition of 0.5 mg/l SPD (9.1 µg/g) compared to the control group (7.1 µg/g) (Fig. 7). However, there was no statistical difference between 1.0 mg/l SPD (7.7 µg/g) and the control group (7.1 µg/g) ($p < 0.05$). Bais *et al.*³⁴ stated that Put and Spd at 1.5 mM concentration separately improved the hairy roots of *Beta vulgaris* and *Tagetes patula* 1.42 and 1.3 folds, respectively, while Spm did not contribute to root growth. In our study, concentrations of different PA types affected the content of rutin accumulation differently and affected the biosynthesis and metabolic effects of these metabolites too differently.

Conclusions

Remarkable results were obtained by culturing Caper leaf explants with different types and concentrations of PAs that enhanced quercetin and rutin contents which are essential phenolic compounds in Caper plant. Another considerable finding was that the regeneration capacity of Caper calli increased in all PA treatments and was found very effective for further studies on micropropagation of Caper plant. It was clear that the use of PA positively supports all parameters obtained as a result of plant tissue culture techniques. As this is the first report in the field of study, it is suggested that the

application of PAs as elicitors can enable the production of commercially important metabolites and phytochemicals used in pharmaceuticals, cosmetics, food, and agriculture-based industries. Effective applications of callus cultures as part of plant biotechnology have commercial potential, particularly for the production of phytochemicals for therapeutic purposes, which can complement traditional agriculture on an industrial scale.

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