EUROPEAN ACADEMIC RESEARCH
Vol. IX, Issue 5/ August 2021

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Electro-enhancement of Salicylic Acid Content in Callus Cultures of *Calendula officinalis* L. Plants

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Abstract

Calli of Marigold, Calendula officinalis L. were exposed to electrical shocks of voltages 200, 250, 300, 350, 400 and 500 volts for 5 msec. as short term electrical shocks (STES) and 10 msec. a long term electrical shocks (LTES). Interestingly both types of shocks were improved biological and physiological growth characters of callus. Fresh weights of exposed callus were assessed 21 and 42 days after exposure. Data obtained proved that both treatments increased callus fresh weights up to 3-fold approximately compared to fresh weight of the non-treated callus. The second increase was after 42 days of exposure. General, STES and LTES were enhanced salicylic acid (SA) biosynthesis in marigold calli up to 20-fold compared with its content in the non-shocked callus.

Keywords: Salicylic Acid Content, Callus Cultures of *Calendula officinalis* L. Plants

INTRODUCTION

Electrical shock, is a physical process of inducing transient permeability of the biological membranes by short pulses of electric fields (Weaver and Chizmadzhev, 1996), used to create transient

pores in plant cell membrane (Smith, *et al.*, 2004; Dev, *et al.*, 2000). Although cell wall is an obstacle to these molecules, cell plasmapheresis before electric treatment causes a partial rupture in the cell wall (Aouida, *et al.,* 2003). These physical phenomena caused by pulsed electrical energy have several applications biotechnologies (Sonoda and Takamura 2014) such as in protoplast electrical fusion (AL-Nema and AL-Mallah, 2020). Researchers benefited from these cellular changes in cells in plant tissue culture experiments, they (Rathore and Goldsworthy, 1985a) referred to the increasing of callus growth of tobacco up to 5-fold by passing electric shock currents between the cultures and the medium. Other workers reported an increasing in callus growth, protein and chlorophyll content as callus exposed to electrical shock (Al-Mallah and Salih, 2003). Marigold, *Calendula officinalis* L. is an annually plant (Asteraceae) cultivated for ornamental and medicinal purposes in Europe and Americas (El-Nashar and Asrar, 2016). Phenolic compounds are of great importance for human health (Martins, *et al.*, 2016) and such salicylic acid compound was harvested from Marigold plants (Larçin, *et al.,* 2015). Salicylic acid is the most important of these as containing an aromatic ring with a hydroxyl group or its derivative, found in plants (Busia, 2016). The best-known natural SA derivative is salicin (ß-glucoside salicylic alcohol) which synthesized in the chloroplast (Allasia, *et al.,* 2018). This investigation aimed to demonstrate the role of electrical shock on growth indicators (fresh weight, total proteins) and salicylic acid biosynthesis of marigold callus.

MATERIALS AND METHODS

Callus Cultures production

Cultures of young and active callus were produced from sterilized leaf explant according to a recent protocol, using MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mgL^{-1} of BAP and fortified with 32 gL^{-1} sucrose (Al-Abasi *et al.,* 2018).

Exposing Callus to Electrical Shocks

One-gram sample of callus (5 samples/treatment) were placed in liquid MS medium in a glass chamber of the device (Al-Mallah, 2002). Samples were exposed by pass electrical pulses 200, 250, 300, 350,

400 and 500 volts for 5 mesc. represented short term electrical shock (STES) and for 10 milliseconds at the same conditions represented long term electrical shock (LTES). All shocked calli samples were transferred to sterilized filter paper to removing traces of liquid media. Then transferred to the surface of 20 ml of agar solidified MS medium provided with 32 gL^{-1} sucrose and supplemented with 1.0 mgL^{-1} NAA, 1.0 mgL^{-1} BAP, as well as non-shocked callus samples. Specimens were incubated at 25 $\pm$ 2 ℃ in culture room conditions subsequently. Shocked and non-shocked calli were sub-cultured every 21 days on the same MS medium mentioned above.

Total Proteins Measurement

Total proteins in shocked and non-shocked calli were estimated (Lowry *et, al.,* 1951). One-gram sample of each shocked and non-shocked fresh calli were grinded in prior cooled mortar in the presence of 5 ml of 5% Trichloroacetic acid (TCA) and mashed by pestle. Total proteins were measured at 660 nm wave length (UV Spectrophotometer, UV-1800 SHIMADZU, Japan).

Salicylic acid Extraction and Deconjugation

Extraction and purification of salicylic acid from Marigold calli samples was preformed according to the standard protocol (Allasia *et al.,* 2018) as in below:

Preparation of Samples

Four hundred mg of shocked and non-shocked calli were each transferred to pre-cooled mortar and immersed into 5 ml of liquid nitrogen. Calli tissues were grind at room temperature, then callus powder transferred to 2.0 ml eppendorf tube, immediately 1.6 ml of 70% ethanol was added. Vortexed the samples for 1.0 min., centrifuged at 10000 x*g* for 10 min. at room temperature. Supernatants were transferred to 15 ml sealed tubes. One more time 1.6 ml of 90% methanol was added to the remaining sediment, vortexed again for 1.0 min. for re-extraction. The mixture was centrifuged at the same conditions and supernatant was added to the stock in 15 ml sealed tubes.

Free Salicylic acid Deconjugation

Both clear supernatants and pooled solutions previously produced contain free and conjugate salicylic acid. Two ml of supernatant samples were each transferred to 2.0 ml microcentrifuge tubes to evaporate EtOH and MeOH by warm airstreams for 2.0 h. Remaining supernatants were transferred to microcentrifuge tubes (2.0 ml capacity) and concentrated up to approx. 600 µl by evaporating alcohol by the same method. To the remaining aqueous solution 65 µl of 20% of aqueous TCA (w/v) was added. Then 650 µl of ethyl acetate and cyclohexane 1:1 (v/v) was added, vortexed for 30 sec., and centrifuged for 2.0 min. at 10,000 x*g* for phase separation. The upper organic phase was transferred to a new 2.0 ml Eppendorf tube, aqueous phase re-extracted again with 650 µl of ethyl acetate-cyclohexane mixture, centrifuged for 2.0 min. at 10,000 x*g* for phase separation. The solvents were evaporated for 30-45 min. up to dryness, the dry residue solubilize in 100 µl of 10% aqueous methanol (v/v) containing 0.1% aqueous trifluoracetic acid TFA (v/v), and vortexed for 1.0 min. Finally, the samples became ready to assessed by HPLC. Salicylic acid standard stock solutions were prepared immediately in day of injection into HPLC device as standard sample, accurately weighing 100 mg of pure salicylic acid (BDH-England) transferred to 250 ml volumetric flask, 100 ml of distilled water was added for dissolve.

Detection & Quantification of SA

Salicylic acid was identified and quantified by reverse phase HPLC (High-performance liquid chromatography Sykman-2014-Germany). Twenty microliters of sample were injected in HPLC, separation conditions were carried out using 5 µm C-18 column (250 x 4.6 mm) at 30 °C, aqueous MeOH gradient from 10% (v/v) used as a linear at 1.0 ml/min. of flow rate, fluorometric detector (excitation at 305 nm; emission at 407 nm). Concentrations of salicylic acid were quantified by comparing peak area of standard salicylic sample with peak area detected from calli samples at the same conditions using the standard equation:

$$\mathrm{C}x(\mu\mathrm{g/g}) = \frac{Ax \times \text{total volume of extract (µl)}}{\mathrm{RF}x \times \mathrm{Aref} \times \text{sample weight (g)}}$$

$$\mathrm{RF}x = \frac{\text{peak area of SA } x \text{ (1µg/ml)}}{\text{peak area of reference SA (1µg/ml)}}$$

Cx= concentration of salicylic acid in the callus samples.
Ax = peak area of salicylic acid in the callus samples.
RFx = response factor of salicylic acid in the callus samples.
Aref = peak area of 1 mg/ml of standard salicylic acid

Results

Electrical Shocks Stimulate Callus Biomass

Data proved that exposed callus to electrical shock for 5 msec. (STES) grown on agar solidified MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium enhanced its growth and biomass. Fresh weight of shocked calli reported more than twice fresh weights of non-shocked callus. Generally, all shocks sustained callus growth and increased biomass, and conserved viability and texture style of treated callus (Table 1).

Table 1: Increasing pattern of fresh weight of leaf callus of *Calendula officinalis* L. exposed to short and long term shocks.

Callus Fresh Weight (gm)					
Short Term Electr. Shock (STES)			**Long Term Electr. Shock (LTES)**		
Volts/msec.	**21 d**	**42 d**	**Volts/msec.**	**21 d**	**42 d**
200/5.0	*6.09*	*25.33*	**200/10**	*6.08*	23.38
250/5.0	*5.29*	*26.94*	**250/10**	7.16	29.77
300/5.0	*7.55*	*29.21*	**300/10**	7.22	**32.51**
350/5.0	*7.79*	***29.3***	**350/10**	7.5	**33.97**
400/5.0	*7.24*	*27.15*	**400/10**	**8.34**	**30.0**
500/5.0	***8.21***	*25.2*	**500/10**	7.32	21.14
Non-shocked Callus (cont.)	*2.80*	*15.72*	**Non-shocked Callus (cont.)**	*2.80*	*15.72*

* Values represent the average weights of 5 replicates/treatment.

Similarly, exposed callus to the same shocks but for 10 msec. (LTES) encouraged growth and callus biomass (Table 1). This was reflected in increasing callus fresh weights after 21 days of exposure as compared with fresh weights of non-shocked callus. Average fresh weights of callus was gradually increased at mild voltages steadily with the raising of voltages to achieve high weight at 400 V/10 msec. after 21 days of exposure. But at 42 days after callus exposure a significant increase in callus fresh weights.

In general results pointed out the enhancement role of electrical shock of fresh weight of Marigold callus grown on the

medium MS + 1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BAP. Callus biomass was increased at 42 days after exposing to each shocks 200, 250, 300, 350, 400 and 500 V/5 msec. Mild effect of these shocks also enhanced the production of compact green callus. Yellowish-green of semi-compact callus culture was produced as exposed to the shocks 200 and 250 V/5 msec. respectively (Fig.1. A, B). Visual examinations showed that callus color was deeper and more consistency in its structure. A significant increase of callus biomass at 300 and 350 V/5 msec. (Fig.1. C, D), outperforming of parameters of other treatments. Then decrease stored in the formal, chromatic and volumetric growth indicators (Fig.1. E, F) when voltage was raised to 400 and 500 V/5 msec. respectively. Exposing other group of callus samples to the same shocks but for 10 msec. stimulated growth indicators including size, biomass and physiological behavior as well as chromatic characteristics.

Shocks influence was smooth, not damage callus color, especially at the first few days after exposure, with retention vitality and green color coupled with a rapid increase of biomass, weight and size. Growth was increased dramatically after 42 days of exposure to electrical shock, this was evident in samples exposed to 200 and 250 volts (Fig 1. G, H). A distinguishable increase of callus biomass that covered and filled up the surface of the medium when exposed to 300, 350 and 400 volts (Fig.1. I, J, K). the only treatment that decrease callus weight was 500 volts (Fig.1. L).

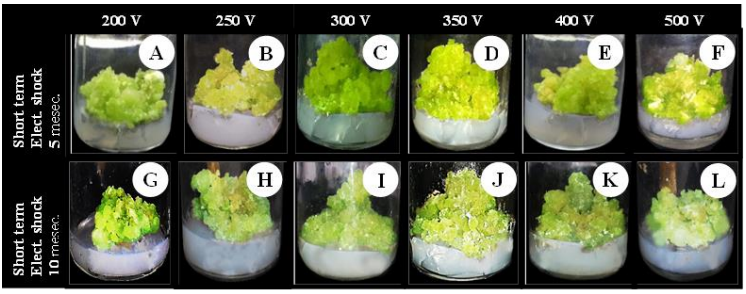

Fig.1: Biomass of *Calendula officinalis* L. callus 42 days after exposure to STES and LTES grown on MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium.

A, B: Semi-compact callus produces as exposed to 200, 250 V/5msec.
C, D: Increase biomass of callus that acquired compact texture exposed to 300, 350 V/msec.
E, F: partial decrease in callus biomass exposed to 400, 500 V/5msec.
GH: Compact and dark green color callus as exposed to 200, 250 V/10 msec.
I, J, K: Normal growth with remarkable increasing in biomass of callus exposed to 300, 350 and 400 V/10 msec.
L: Reduced biomass of callus exposed to 500 V /10 msec.

Stimulation of Protein Content

Data demonstrated that electrical shocks promoted increases in protein contents of the exposed callus to various pulses for 5 msec. (Fig.2 A). Estimation of protein contents showed that the gradual increase was began from 200 V/5 msec. and reached the maximum value 287.89 µg gm^{-1} with callus exposed to 350 V. Total protein contents were decreased gradually when voltage raised up to 400 and 500 V/5 msec., respectively.

Observations showed that subsequent increases of protein were achieved with voltage increase. The highest increase in proteins recorded 383.25 and 392.08 µg mg^{-1} at 300, 350 volts/10 msec. respectively. It was found that the shocks 400 and 500 volts/10 msec. decreased protein contents in treated callus (Fig. 2. B).

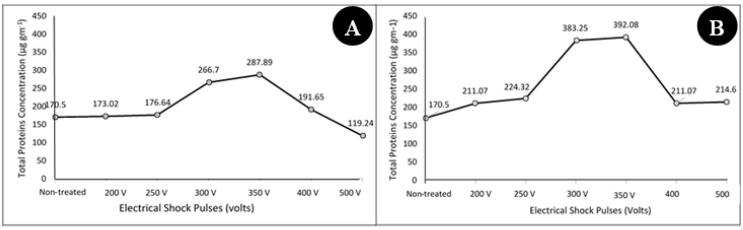

Fig. 2: Protein contents in callus of *Calendula officinalis* L. grown on MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium 42 days after electrical shocks.
A: Protein contents in callus exposed to various shocks for 5 msec.
B: Proteins content in callus exposed to the same shocks in (A) for 10 msec.

Accumulation of Salicylic Acid in Shocked Callus

The results (Table 2) confirmed the presence of SA in Marigold callus after exposed to each of STES (200, 250, 300, 350, 400, 500 /5 mesc.), that grown in the medium (MS + 1.0 mg^{-1} NAA + 0.1 mg L^{-1} BAP). Results indicate the positive influence of electrical shocks in increasing callus growth and its biomass was reflected in high content of salicylic acid. Whereas in non-shocked callus low content of salicylic acid was recorded. Gradual increase of salicylic acid occurred as voltage raised and the highest content 105.38 µg gm^{-1} was in callus exposed to 350 volts/5 msec.

Table 2: Enhancement of salicylic acid in Calendula officinalis L. callus, exposed to different electrical shocks for 5 mesc., grown in MS + 1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BAP medium.

Treatments		Peak area	SA Conc. (µg/g)
Callus exposed to electric shock	**200 V/5 msec.**	1319.692	18.46
	250 V/5 msec.	1625.749	22.74
	300 V/5 msec.	4286.694	59.98
	350 V/5 msec.	7531.537	**105.38**
	400 V/5 msec.	4267.167	59.71
	500 V/5 msec.	1725.75	24.14
Un-shocked callus		*359.327*	*5.02*
Standard SA (cont.)		*7147.510*	100

High performance liquid chromatography showed that callus exposed to each of shocks 200, 250, 300, 350, 400, 500 V/5 msec. as well as curves of both un-shocked and standard samples proved the presence of salicylic acid in all callus samples. Levels of salicylic acid in callus extracts samples was affected by electrical shocks in a way that led to an increase of quantity for many folds compared to untreated callus. Firstly, standard samples of salicylic acid were injected involved 3.424 min. (Fig. 3 A). Then, un-shocked callus extract sample was injected and SA curve was coinciding with the retention time in standard sample (Fig. 3 B), and followed by injection of the electrical shocked callus extracts samples, respectively. Curves data showed the variation of SA level in callus samples exposed to electric shock at 200 and 250 volts shown the starting increase of SA compared to un-shocked callus sample (Fig. 3 C, D) respectively. Subsequently, SA level was increased twice in callus sample exposed to 300 V as compared with other treatments (Fig. 4 E). In sample exposed to 350 volts was found that SA content was equal or higher than the standard sample (Fig. 3 F), and levels of SA were decreased in samples exposed to shocks of 400 and 500 volts (Fig. 3 G, H) respectively.

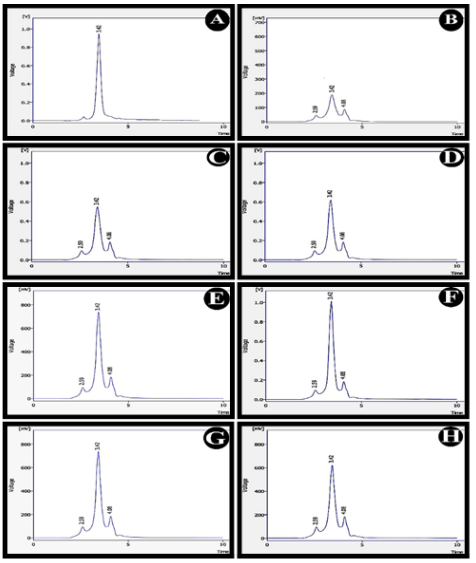

Fig. 3: Measurements of Salicylic acid content in *Calendula officinalis* L. callus 42 days after exposure to various electrical shocks.

A: Standard sample of Salicylic acid 100 µg gm^{-1}.

B: Non-shocked callus sample.

C, D, E: High peaks resulted from exposed callus to 200, 250 and 300 V/5 msec., respectively, note the peak height.

F: Large size and elevate of callus sample curve exposed to 350 V/5 msec. referred to high level of SA.

G, H: Partial reduction in peak height in samples exposed to shocks at 400 and 500 V/5 msec., respectively.

Enhancement of Salicylic Acid

The results (Table 3) clearly showed that the enhancement role of electrical shocks for 10 msec. on SA biosynthesis was significantly variable in its concentrations 42 days after exposure. Results confirmed that callus samples exposed to 200 V. stimulate increasing of SA content. This treatment resulted in four-fold increase of SA content compared to quantity in the non-shocked samples. Raising voltages to 250 volts, sustained the amount of SA. Significant increase was achieved in samples exposed to 300 V. Interestingly, highest value of SA 111.83 µg gm^{-1} was obtained from callus exposed 350 V. SA concentrations were gradually decreased in samples exposed to 400 and 500 volts respectively.

Table 3: Enhancement of salicylic acid in Calendula officinalis L. callus, exposed to different electrical shocks for 10 mesc., grown in MS + 1.0 mg L -1 NAA + 0.1 mg L-1 BAP medium.

Treatments		Peak area	SA Conc. (µg/g)
Callus exposed to electric shock	**200 V/10 msec.**	1625.749	22.74
	250 V/10 msec.	1866.118	26.11
	300 V/10 msec.	6383.337	83.32
	350 V/10 msec.	7992.226	**111.83**
	400 V/10 msec.	3361.937	47.04
	500 V/10 msec.	2366.085	33.10
Un-shocked callus		*359.327*	*5.02*
Standard SA (cont.)		*7147.510*	100

According to standard salicylic acid curve from HPLC output (Fig. 4. A), curves of exposed calli samples after 42 days and non-exposed that grown on agar solidified medium MS + 1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BAP were exactly involved the same retention time of the standard curve. Figure (4. B), this express the presence of SA in un-shocked callus sample, whereas, (Fig.4. C) recorded the height curve expressing the presence of SA and its concentration in exposed callus to electric shock of 200 V/10 mesc. Similarly curve height was increased in exposed callus to electric shock of 250 V/1 mesc. (Fig.4. D), and 300 V/10 mesc. (Fig. 4. E). The highest elevation of curve of SA concentration expressed was obtained in callus sample exposed to 350 V/10 mesc. which exceeded the height of standard sample curve (Fig. 4. F). Continuing lifting voltages up to 400 and 500 volts caused to inhibition of both build-up and accumulation of SA in the callus samples (Fig.4. G, H) respectively.

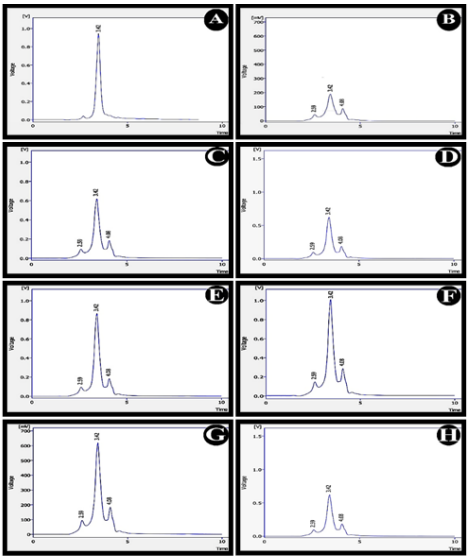

Fig. 4: Accumulation of Salicylic acid in *Calendula officinalis* L. callus 42 days after exposure to electrical shock for 10 msec.

A: Standard sample of Salicylic acid 100 µg gm^{-1}.

B: Non-shocked callus sample.

C, D, E: Peak height increase of curves of calli samples exposed to 200, 250 and 300 volts, respectively.

F: Great size of callus sample curve promoted by exposed to 350 V. because enriches of SA concentration.

G, H: Fractional reduction in peak height in samples exposed to shocks at 400 and 500 V/5 msec., respectively.

DISCUSSION

Stimulation of callus growth by application of electric pulses might due to either auxins movement that described as a polar orientate of callus growth in such direction (Rathore and Goldsworthy, 1985b), or to the increase of permeability of cells walls that support formation of meristem cells since more nutrients was uptake from culture media (Davey and Anthony, 2010). Many investigators attributed the increasing of callus growth to the cell content increase of nucleic acids and proteins needed for cellular division process that encouraged some physiological function that reflected on increasing fresh weights and protein contents (Al-Mallah and Salih, 2003). In this study, the enhancement of callus growth may explain to a primary sites of action at plasma-membrane level involving changes of electric potential or in enzymes activity. Other models that suggest disturbance in the metabolic machinery of cells including proteins and/or genomic

molecules of DNA and RNA (Rovelli and Bennici, 2000). The dark green color of callus probably attributed to the increases content of chlorophyll, which required in uptake of extra amount of mineral elements necessary in synthesis of chlorophyll molecules (Black, *et al.,* 1971). Increasing of total proteins may be explained to the role of electrical pulses that causing changes in enzymes activity such as peroxidase that considered a strong indicator, in many plant species, for protein increase reflected positively in increasing cellular activity including the increasing that size and weight of cells (Suleiman *et al.,* 2018).

In this study, electro-enhancement of salicylic acid might due to irritation state in exposed plant cells (Blackman and Overall, 1998). Accordingly, since SA influenced cell membrane permeability and ion fluxes across them such as K^+ and Ca^{+2} and H_2O_2. It was observed that conversion of benzoic acid to salicylic acid requires the presence of hydrogen peroxide (H_2O_2). Moreover, many researchers descripted that the explanation of this situation is complex and contradictory, because of initial elevation of calcium level near the membrane can trigger membrane depolarization (Hayat *et al.,* 2013). Thereafter, ionic channels are activated either by electric activity exposure or change in the content of free Ca^{+2} and H_2O_2 in the cytosol. This is may be a key signal for many regulatory processes and subsequent pathway of calcium signal transmission include hormones (jasmonic acid and salicylic acids) activities. Finally, salicylic acid synthesis may have enhanced also because an increase in H_2O_2 content. This is similarly occurred in tobacco (Neuenschwander *et al.*, 1995) and *Arabidopsis thaliana* plants (Summermatter *et al.,* 1995).

Acknowledgments

The others are very grateful to the University of Mosul/ Colle. of Agric. and forest. and Colle. of Edu. for Pure Sci. for proving facilities, which allowing researchers to carry out this work in biotechnology and genetic manipulation labs in department of biology. Thanks due to Mr. Ali Al-Tamimi, Ministry of Sci. and Techno. lab. for his assistance in HPLC works.

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