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The effects of elicitors and precursor on *in-vitro* cultures of *Trifolium resupinatum* for sustainable metabolite accumulation and antioxidant activity

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ABSTRACT

Natural products are being searched for the potential source of medicines and drugs. *Trifolium resupinatum* is a significant hay crop in cold regions of Iran, Iraq, and Middle East Asia. In this study, assessment of the property of elicitors and precursors on the antioxidant ability of *T. resupinatum* and on the capitulate improvement of metabolites were done aiming to advance the metabolite and antioxidant production. Explants and containerized plants of *T. resupinatum* were cultivated before treatment with elicitors and precursors. Plants grown from different explants and culture conditions such as somatic embryos, shade-grown, dark-induced callus, and light-induced callus were treated with different concentrations of elicitors and precursors. The variations in the biosynthesis of secondary metabolites were tissue and environment-specific. Though there was similarity in the relation of content with the concentration of elicitors and precursors shows tissue specificity. The results showed that Methyl Jasmonate (MJ), Salicylic acid (SA), and Glutathione (Glu) were effective on treatments on the bio-production of phenolic and flavonoid compounds. The overall results indicate significant effects of treatments in improving the shade-grown plant activity. The correlation between phenolic and flavonoid content was statistically evaluated with antioxidant activity. Enzymatic activity was influenced by all three effectors and precursors; it was quite more under the influence of SA. The current study displayed the enhancement of metabolites (phenolic and flavonoid) production and increase of antioxidant and enzymatic activities, particularly in the presence of glutamine as precursors and elicitors.

1. Introduction

In the age of emerging new disease and increasing antibiotic resistance, the natural products are being searched for a potential source of medicines and drugs. In a survey of pharmacopeias, up to 25% of all medicines are originated from natural sources, including herbs, underutilized plants, and flowering plants (Eisenman et al., 2012). *T. resupinatum* popularly known as clover and it is native to central and southern Europe, Mediterranean, and southwest Asia (Sabudak et al., 2008; Sabudak and Guler, 2009). *T. resupinatum* forms dense swards of rosette growth with a hollow stem, having pink to violet flower, once matured it would have white woolly seed head (Simões et al., 2004). It provides high nutritive pasture of palatable hay used for fodder. Its high moisture and protein make it inappropriate for ensiling. Apart from providing fodder, it is N-fixing legume, helps in augmented ammonia making in the rumen to the soil (Molle et al., 2008). In Asian sub-continental *T. resupinatum* (clover) is regarded as a weed which has an allelopathic effect on germinating weeds (Maighany et al., 2007; Tungmunnithum et al., 2018) although the efficiency is not confirmed by field trials. Much work has been done in Great Britain, and India showed that protein from leafy plants gives a maximum yield of protein and that edible Protein could be easily extracted on a large scale from these crops (Gordon, 1996; Leutner et al., 2001; Malaviya et al., 2005; Matkowski, 2008). Flavonoids and phenolic acids are found in all plant parts and possess antioxidant activities (Lee et al., 2001; Wong et al., 2006; Benjapak et al., 2008; Andarwulan et al., 2010; Andarwulan et al., 2012). The addition of elicitors, and/or precursors increases antioxidant activity(Lila et al., 2005). Jasmonate (MJ) and Salicylic acid (SA) are effective elicitors, triggers the signal transduction path in plants causing enhanced production capacity in plants (Lila et al., 2005). *T. resupinatum*

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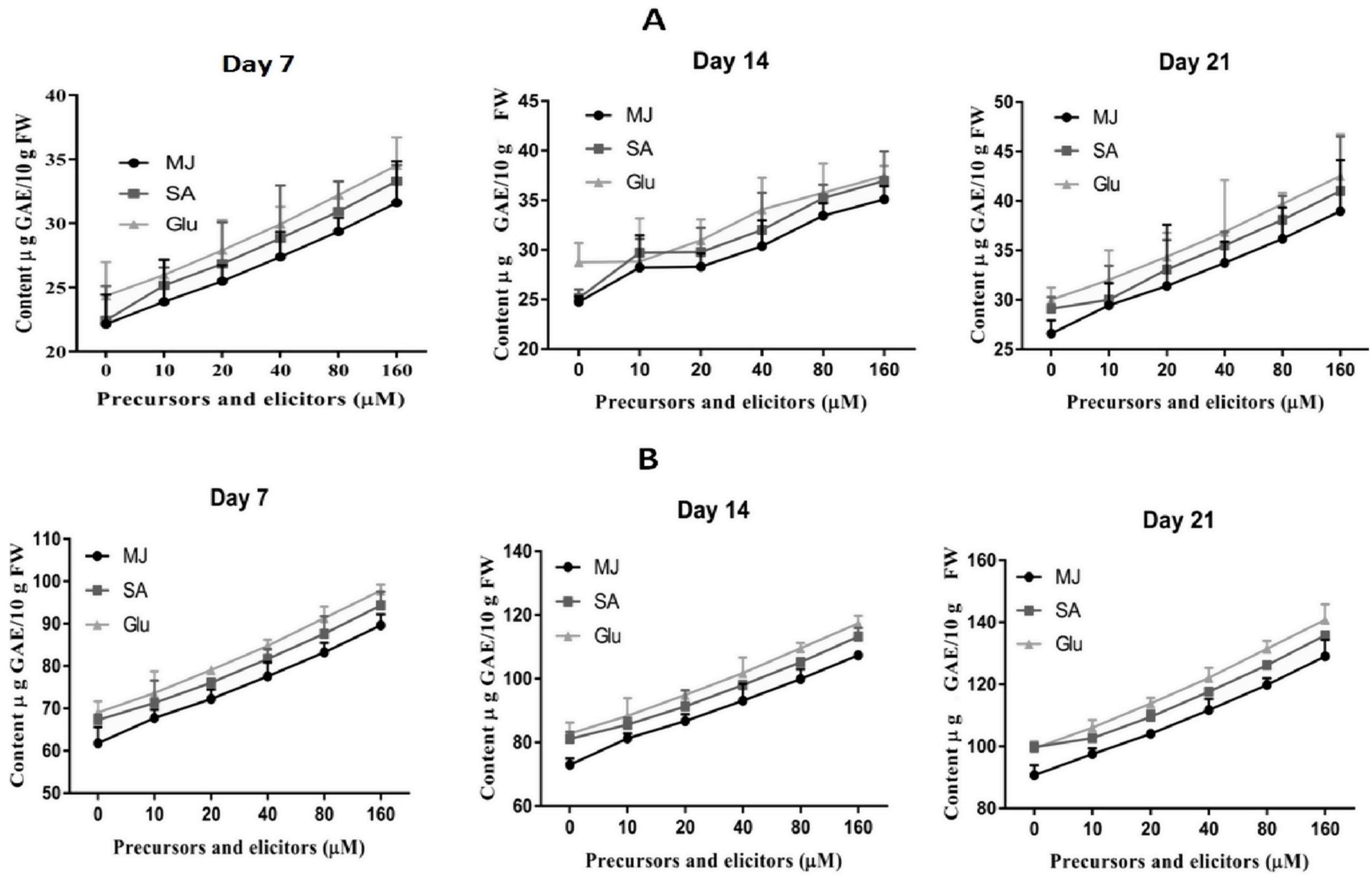

Fig. 1. Determination of total phenolic (A) and total flavonoid contents (B) of shade-grown plants after treatment. Total phenolic content (μg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD).Total flavonoid content (μg rutin equivalent (RE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Duncan's multiple range test was used to determine a significant difference ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is rich in protein and nutrition and makes it an important source of enzymes which may have some kind of oxidative enzymes leading to stress responses (Kassem et al., 2017). To the finest up to our knowledge, we found that no research and/or comparative work has been done on the metabolite production and antioxidant effects of *T. resupinatum* under influence of elicitors and precursor. The present study evaluates the property of elicitors and precursor on the yield enhancement of *T. resupinatum* metabolites and its antioxidant and enzymatic capacity in a different type of tissue cultures.

2. Materials and methods

2.1. Plant materials

T. resupinatum were collected from local vendors, Baghdad city and identification was confirmed by the botanist of Mustansiriyah University. Mass propagation was done shade house and the plant materials were used for the elicitor and precursor treatment. Nodal cuttings of stems were used as explants placed in serial rows so that the shoot acquires a depth. For the next one month, continuously misting was done for 1–2 min during the daytime, to maintain moisture and high relative humidity. Light intensity was reduced to half of ambient (Pacholczak et al., 2005). Once there was a growth of nursery about 5 cm, it was gradually shifted to ambient conditions for few days before establishing in new pots for further shoot expansion. The explants grown sufficiently were cultivated further in the shade house prior to exposure of SA (abiotic elicitor), MJ (abiotic elicitor) or Glutathione (Glu) (precursor) respectively.

2.2. In vitro cultures

T. resupinatum cultures were propagated in various conditions i. e−shade housed; somatic embryogenic cultures, light-induced callus cultures, and dark-induced callus at plant tissue culture laboratory, Biology department, Mustansiriyah University, Iraq before elicitation process was performed using Glu, MJ and/or SA separately. The shoot was inducted from nodal explants that were inoculated on MS (Murashige-Skoog) medium supplemented with 2.0 mg/l of 6-Benzylaminopurine (BAP), 0.5 mg/l Indol Acetic Acid (IAA), 3.0% (w/v) sucrose and 0.25% (w/v) Phytage and pH 7.0) as per protocol following skeptical methods. Inoculated explants were kept under the following conditions 25 ± 1 °C temperature, 16-h photoperiod. The adventitious shoot came out from explants were cultured again in fresh MS medium with four weeks interval to get multiplied explants as many as possible. This was done for light-induced callus but for dark-induced callus, the grown callus was kept under dark all time. The grown calli were used to subculture into fresh MS medium once in a month for mass multiplication (Prodhan et al., 2016; Hasan et al., 2019). This step was repeated for three-times and each case calli were transferred to 50 ml of MS medium containing 3.0% (w/v) sucrose, 2.0 mg/l of 1-Naphthaleneacetic acid (NAA) and 1.0 mg/l kinetin (Kin) for inducting globular somatic embryo after three weeks of inoculation.

2.3. Elicitors and precursors

To achieve enhanced and maximum yield of secondary metabolites from plants efforts have been given to find the potent elicitors and precursors and MJ, SA and Glu were used to conduct this study. All these

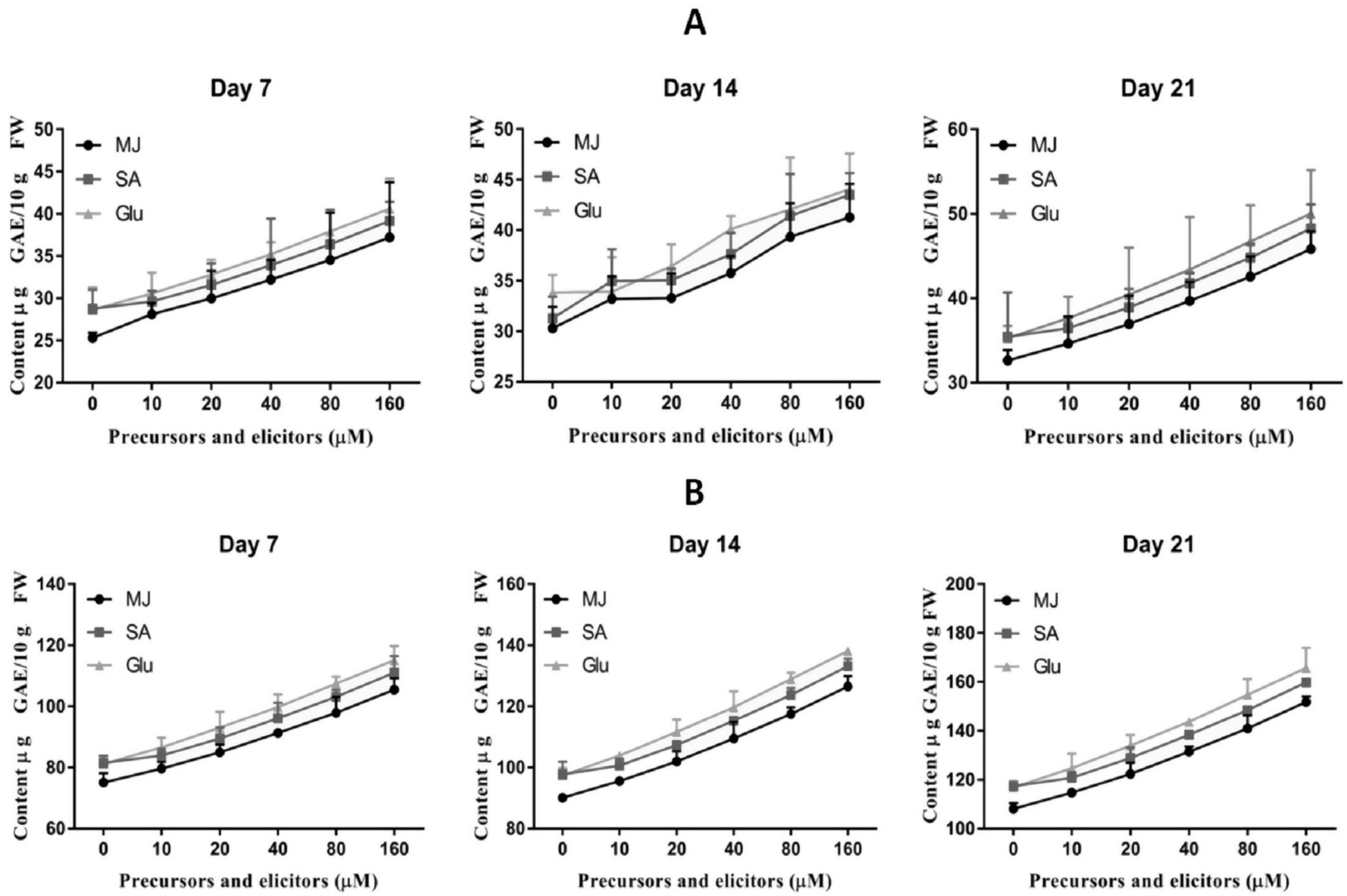

Fig. 2. Determination of total phenolic (A) and total flavonoid contents (B) of somatic embryos after treatment. Total phenolic content (µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Total flavonoid content (µg rutin equivalent (RE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

stock solutions were prepared in 100 µM of 95% of ethanol, sterilized using 0.2 µm syringe filter in the laminar flow cabinet and stocked at 4 °C until required. Elicitors and precursors- MJ, SA and Glu were used to evaluate the production ability of light-induced, dark-induced and somatic embryos of *T. resupinatum* (treated individually with elicitors and precursors). Explants were treated with different concentrations of precursors and elicitors (0, 10, 20, 40, 80, 160 µM) for different periods (0, 1, 2, 3, 4 weeks).

2.4. Treatment of *T. resupinatum* with elicitors and precursors

A working solution was prepared for each stock before it can be used to acquire the desired concentration. In this experiment, the working solution was sprayed on shade-grown 6 month old individual plant with varying concentrations of all three MJ, SA or Glu. Plants sprayed with distilled water were taken as control, in this experiment. The spraying was done at early morning hours by manual methods and the volume of spray was made sufficient enough till it covers plant completely till it dripped. Steps were followed by covering the plant with a vinyl pack for 1–2 h, and then treated plants were isolated and washed and left in open place until the remaining elicitors and precursor were washed away. At an interval of seven days, a time course study on metabolite production was conducted to see the influence of elicitors and precursors for 3-weeks (Królicka et al., 2008).

2.5. Plant extraction and estimation of secondary metabolites

According to a published protocol of Andarwulan et al. (2010), Secondary metabolites of the experimental plant were extracted and quantified from in vitro cultures of *T. resupinatum*. In brief, the plants were washed, cleansed and nicely blotted dried before preparing the crude extract. With the use of motor and pestle, a 50 g fresh plant sample made paste was inoculated into 25 ml of 75% (v/v) aqueous methanol (HPLC grade). In solution 0.5 g/l of TBHQ was used as an antioxidant and 1 ml of 1.0 M HCl was added to release the bound flavonoids by hydrolyzing the glycosides. Each individual sample was refluxed at 92 °C for 2-h and cooled on ice immediately. The procedure was repeated for three times before it was washed through 0.45 µm filter paper (Whatman, England) and stocked at −20 °C till further use.

2.6. Determination of total phenolic content

The phenolic content was quantified following the earlier described protocol (Hidayathulla et al., 2018) based on the Folin Ciocalteau method. In a sample extract (50 µL), 2 mL of sodium carbonate (2%) was added and kept without disturbing for another 5 min. In the end, 100 µL of Folin Ciocalteau reagent was mixed with reaction mixture and left undisturbed for 30 min at room temperature. The readings of the mixture were taken at 700 nm. A blank with gallic acid was used as a control standard for the calibration curve preparation. The phenolic content of the extract was articulated as gallic acid equivalents (µg GAE/10 g FW).

2.7. Determination of total flavonoid content

The flavonoid content of extracts sample was determined by

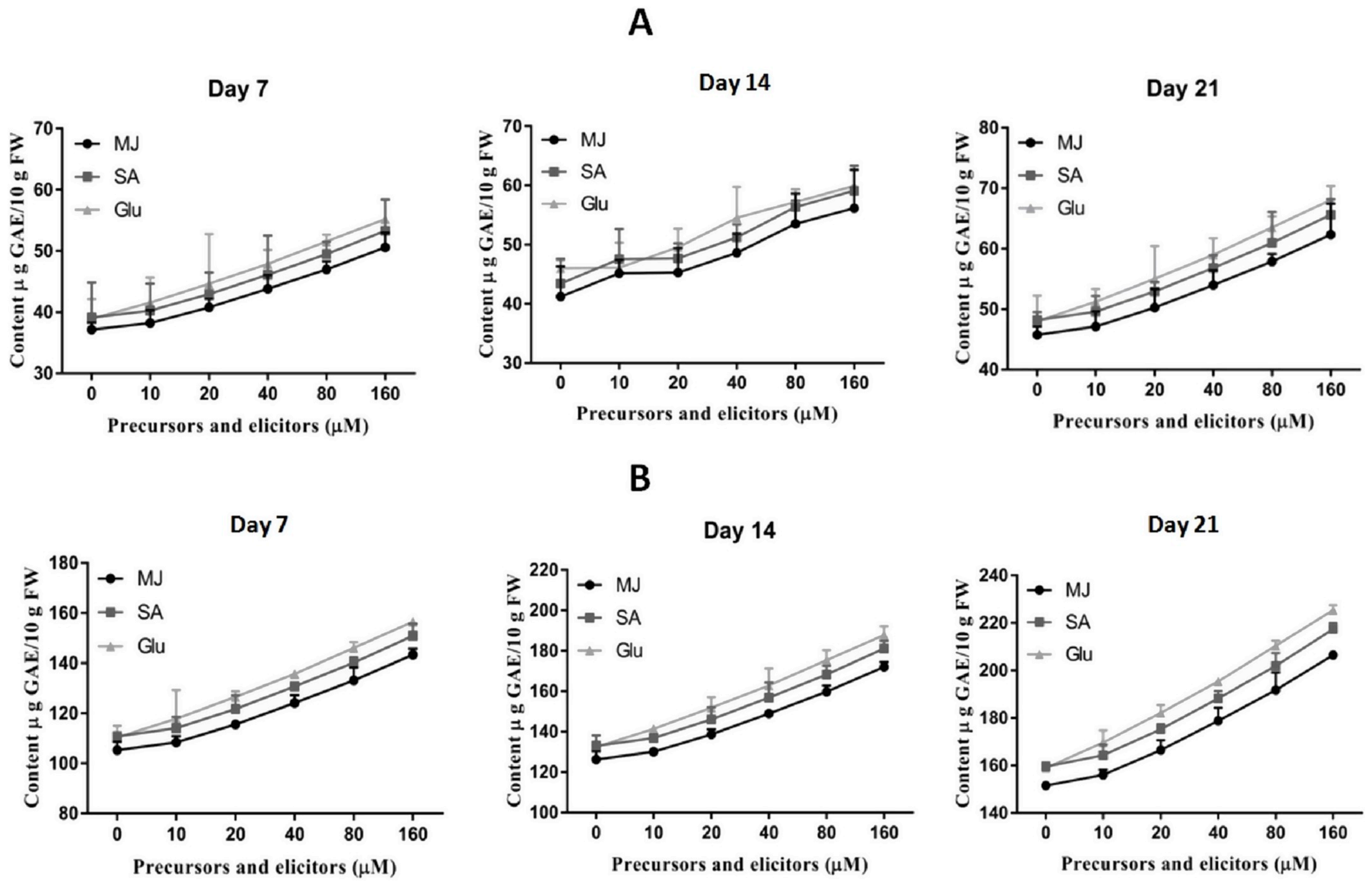

Fig. 3. Determination of total phenolic (A) and total flavonoid contents (B) of light-induced callus after treatment. Total phenolic content (μg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Total flavonoid content (μg rutin equivalent (RE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

following the steps as mentioned in Hidayathulla et al., (2018). One ml of extract was mixed with a methanol solution (1 ml) containing aluminum chloride (2%) which results in formation of flavonoid-aluminum complex during 15min incubation at room temperature. The intensity of this complex indicates the flavonoid content as measured at 425 nm by using a UV–Vis spectrophotometer. The total flavonoid content was expressed as the rutin equivalent (μg RE/10 g FW) in mg/g of the dry weight of the extract.

2.8. Determination of antioxidant activities

Diphenyl-2-picrylhydrazyl (DPPH) was utilized to measure the antioxidant activity based on the scavenging action of DPPH free radical. This was determined following the steps as mentioned by Hidayathulla et al., (2018) previously. In brief, DPPH accepts an electron or hydrogen to produce a pale violet diamagnetic molecule which gives rise to pale violet when mixed with DPPH. The absorbance of the color measured indicates the antioxidant activity of the extract at 520 nm after 30min; percent inhibition is calculated as mentioned in the literature (Di Ilio, Polidoro et al., 1983).

2.9. Ferric reducing antioxidant potential (FRAP) assay

The antioxidant activity was calculated by the ferric reducing antioxidant power assay (FRAP), which depends on their ability to form the complex with iron ion. In the presence of this complex formation, the absorbance of the solution increases indicating the antioxidant activity. Sample's OD was taken at 700 nm using UV-Spectrophotometer (Benzie and Strain, 1996).

2.10. Determination of enzymatic activities

2.10.1. Assay of Glutathione peroxidase activity

GST peroxidase activity was measured using the protocol of an earlier published article which uses the consumed NADPH to estimate Glutathione peroxidase activity. Conversion of NADPH to NADP is monitored using spectrophotometer at 340 nm absorbance after initiating the reaction. The true enzyme activity reported as micromoles of NADPH per minute per 109 cells.

2.10.2. Assay of glutathione reductase' activity

The glutathione reducatase estimation was done based on spectrophotometric assay estimating it's reductase activity to reduced glutathione disulfide to glutathione which was indirectly measured by monitoring the oxidation of NADPH indicated by change the color of solution at 340 nm (Mannervik, 1999).

2.10.3. Assay of glutathione S-transferase (GSH-ST) activity

Glutathione S-transferase (GSH-ST) activity was measured following the protocol of Habig and Jakoby (1981) where chlorodinitrobenzen was used as substrate (Habig and Jakoby, 1981). GSH-chlorodinitrobenzene conjugate is formed during the reaction process and the change in absorbance is measured at 340 nm which indicate the GSH-ST activity (Di Ilio, Polidoro et al., 1983).

2.11. Statistical analysis

For each of the estimation there were five biological replicates were used and each repeat was indicative of one new plant explants. Three

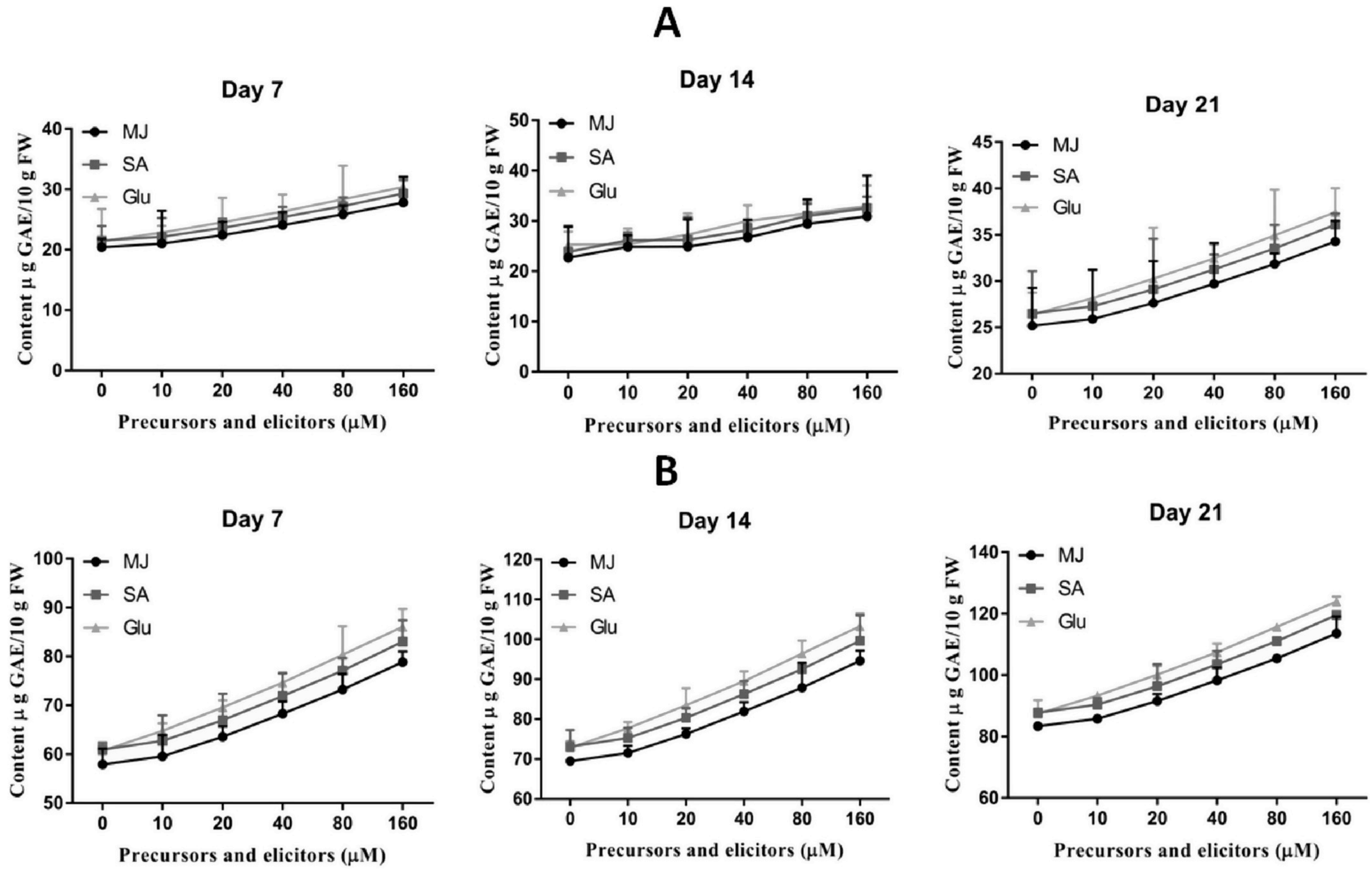

Fig. 4. Determination of total phenolic (A) and total flavonoid contents (B) of dark-induced callus after treatment. Total phenolic content (µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Total flavonoid content (µg rutin equivalent (RE) per 10 g fresh weight samples (FW) ± Standard Deviation (SD). Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

Table 1
Determination of effect of MJ, SA and Glu on the DPPH radical scavenging activity and FRAP reducing activity (shade-grown plant).

Elicitors/precursors	Concentration (µM)	DPPH radical scavenging activity (%)± SD			FRAP assay (µg FeSO4/10 g FW) ± SD		
		7th day	14th day	21st day	7th day	14th day	21st day
MJ	0	37.85 ± 1.23	42.16 ± 2.46	46.38 ± 1.36	191.76 ± 3.30	210.94 ± 3.15	232.03 ± 3.51
	10	40.33 ± 3.51	44.36 ± 4.53	48.79 ± 3.52	201.75 ± 7.45	221.92 ± 4.51	244.11 ± 2.48
	20	44.78 ± 4.42	49.26 ± 1.35	54.19 ± 5.15	224.05 ± 8.13	246.46 ± 2.44	271.10 ± 4.52
	40	46.76 ± 2.38	51.44 ± 5.41	56.58 ± 2.64	233.94 ± 1.59	257.33 ± 5.22	283.07 ± 6.25
	80	48.67 ± 1.64	53.54 ± 3.24	58.89 ± 4.32	243.50 ± 4.64	267.85 ± 1.35	294.64 ± 1.44
	160	49.97 ± 6.22	54.96 ± 1.52	60.46 ± 1.25	249.99 ± 5.35	274.98 ± 4.54	302.48 ± 5.53
SA	0	42.59 ± 2.15	46.85 ± 4.45	51.53 ± 2.54	213.07 ± 2.24	234.37 ± 3.63	257.81 ± 2.46
	10	44.81 ± 5.46	49.29 ± 4.84	54.21 ± 5.42	224.16 ± 1.52	246.58 ± 6.26	271.24 ± 4.22
	20	49.76 ± 4.23	54.74 ± 6.48	60.21 ± 3.25	248.95 ± 4.44	273.84 ± 5.58	301.22 ± 1.45
	40	51.96 ± 2.52	57.15 ± 3.55	62.87 ± 5.63	259.93 ± 4.15	285.93 ± 4.44	314.52 ± 2.54
	80	54.08 ± 1.45	59.49 ± 5.14	65.44 ± 6.20	270.56 ± 1.21	297.61 ± 1.22	327.38 ± 5.22
	160	55.52 ± 8.24	61.07 ± 1.22	67.18 ± 5.54	277.76 ± 5.34	305.54 ± 8.15	336.09 ± 4.15
Glu	0	53.24 ± 2.11	58.56 ± 2.51	64.41 ± 2.42	266.33 ± 3.52	292.97 ± 4.54	322.26 ± 1.41
	10	56.01 ± 1.32	61.61 ± 4.44	67.77 ± 1.81	280.20 ± 5.24	308.22 ± 2.42	339.05 ± 2.54
	20	62.20 ± 4.65	68.42 ± 5.25	75.26 ± 2.34	311.18 ± 4.11	342.30 ± 1.24	376.53 ± 5.22
	40	64.94 ± 2.20	71.44 ± 1.12	78.58 ± 3.12	324.91 ± 5.55	357.41 ± 6.12	393.15 ± 4.45
	80	67.60 ± 3.55	74.36 ± 2.56	81.79 ± 6.25	338.20 ± 2.43	372.02 ± 3.48	409.22 ± 3.64
	160	70.25 ± 5.44	77.28 ± 4.32	85.01 ± 2.34	351.48 ± 1.26	386.63 ± 2.30	425.29 ± 1.31

*Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

technical and identical replicates were used to estimate the average of that particular experiment. Data were subjected to Social Sciences (SPSS) software version 16. Considerable differences among the treatments were determined by analysis of variance (ANOVA); Duncan's multiple range tests (DMRT) and Pearson's correlation coefficient test to measure the correlations of secondary metabolites manufacture and its antioxidant and enzymatic activities.

3. Results

3.1. Determination of total phenolic and total flavonoid content

The variation in the biosynthesis of secondary metabolites was tissue and environment specific. Though there was similarity in the relation of content with the concentration of elicitors and precursors show tissue

Table 2
Determination of effect of MJ, SA and Glu on the DPPH radical scavenging activity and FRAP reducing activity (somatic embryos).

Elicitors/precursors	Concentration (μM)	DPPH radical scavenging activity (%)± SD			FRAP assay (μg FeSO4/10 g FW) ± SD		
		7th day	14th day	21st day	7th day	14th day	21st day
MJ	0	42.59 ± 3.25	46.85 ± 8.31	51.53 ± 3.30	213.07 ± 3.31	234.37 ± 3.13	257.81 ± 3.31
	10	44.81 ± 4.33	49.29 ± 4.23	54.21 ± 5.14	224.16 ± 5.22	246.58 ± 5.21	271.24 ± 5.53
	20	49.76 ± 2.45	54.74 ± 2.52	60.21 ± 4.42	248.95 ± 4.53	273.84 ± 1.55	301.22 ± 4.45
	40	51.96 ± 6.12	57.15 ± 5.11	62.87 ± 2.25	259.93 ± 2.45	285.93 ± 6.34	314.52 ± 2.24
	80	54.08 ± 5.50	59.49 ± 6.52	65.44 ± 5.51	270.56 ± 5.12	297.61 ± 5.52	327.38 ± 1.52
	160	55.52 ± 1.23	61.07 ± 3.25	67.18 ± 6.45	277.76 ± 1.45	305.54 ± 3.44	336.09 ± 5.21
SA	0	47.32 ± 4.11	52.05 ± 5.44	57.26 ± 3.12	236.74 ± 5.34	260.42 ± 2.26	286.46 ± 3.55
	10	49.78 ± 2.42	54.76 ± 4.22	60.24 ± 5.24	249.07 ± 4.52	273.98 ± 5.83	301.37 ± 4.63
	20	55.29 ± 5.29	60.82 ± 1.55	66.90 ± 4.53	276.61 ± 2.25	304.27 ± 4.62	334.69 ± 4.25
	40	57.73 ± 4.53	63.50 ± 7.26	69.85 ± 2.35	288.81 ± 5.51	317.69 ± 6.55	349.46 ± 2.51
	80	60.09 ± 2.50	66.10 ± 4.12	72.71 ± 8.64	300.62 ± 4.23	330.68 ± 9.44	363.75 ± 4.14
	160	61.69 ± 3.18	67.86 ± 2.55	74.64 ± 4.41	308.63 ± 2.40	339.49 ± 8.22	373.44 ± 1.26
Glu	0	59.15 ± 9.32	65.07 ± 4.34	71.57 ± 2.22	295.93 ± 5.64	325.52 ± 5.55	358.07 ± 8.64
	10	62.23 ± 5.52	68.45 ± 1.52	75.30 ± 5.58	311.34 ± 4.59	342.47 ± 2.82	376.72 ± 3.38
	20	69.11 ± 3.11	76.02 ± 3.45	83.62 ± 2.24	345.76 ± 2.28	380.33 ± 4.45	418.37 ± 5.41
	40	72.16 ± 4.34	79.38 ± 6.24	87.31 ± 4.42	361.02 ± 6.12	397.12 ± 3.52	436.83 ± 4.22
	80	75.11 ± 2.21	82.62 ± 5.52	90.88 ± 3.63	375.78 ± 3.84	413.35 ± 5.15	454.69 ± 2.56
	160	77.11 ± 6.40	84.82 ± 4.65	93.30 ± 2.66	385.78 ± 5.52	424.36 ± 4.41	466.80 ± 5.22

*Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

Table 3
Determination of effect of MJ, SA and Glu on the DPPH radical scavenging activity and FRAP reducing activity (Light induced).

Elicitors/precursors	Concentration (μM)	DPPH radical scavenging activity (%)			FRAP assay (μg FeSO4/10 g FW)		
		7th day	14th day	21st day	7th day	14th day	21st day
MJ	0	47.27 ± 3.31	52.00 ± 1.83	57.20 ± 4.35	236.51 ± 53	260.16 ± 6.36	286.17 ± 3.60
	10	49.73 ± 1.43	54.71 ± 3.44	60.18 ± 2.12	248.82 ± 35	273.70 ± 8.54	301.07 ± 5.25
	20	55.23 ± 2.15	60.76 ± 5.11	66.83 ± 1.14	276.33 ± 51	303.96 ± 4.12	334.36 ± 4.52
	40	57.67 ± 5.82	63.44 ± 1.68	69.78 ± 7.85	288.52 ± 84	317.38 ± 4.48	349.11 ± 2.44
	80	60.03 ± 1.45	66.03 ± 2.53	72.63 ± 5.21	300.32 ± 62	330.35 ± 2.25	363.39 ± 8.68
	160	61.63 ± 2.21	67.79 ± 5.45	74.57 ± 4.54	308.32 ± 28	339.15 ± 5.53	373.06 ± 6.36
SA	0	52.53 ± 5.52	57.78 ± 3.24	63.56 ± 2.45	262.78 ± 44	289.06 ± 5.46	317.97 ± 3.83
	10	55.26 ± 3.65	60.79 ± 5.49	66.86 ± 4.52	276.47 ± 22	304.11 ± 5.14	334.53 ± 515
	20	61.37 ± 5.32	67.51 ± 4.51	74.26 ± 6.53	307.03 ± 55	337.74 ± 2.61	371.51 ± 4.22
	40	64.08 ± 2.25	70.49 ± 3.13	77.53 ± 3.66	320.58 ± 14	352.64 ± 4.55	387.90 ± 2.59
	80	66.70 ± 5.72	73.37 ± 2.25	80.70 ± 2.24	333.69 ± 41	367.06 ± 5.22	403.76 ± 5.45
	160	68.47 ± 2.55	75.32 ± 1.44	82.85 ± 5.41	342.57 ± 86	376.83 ± 2.54	414.51 ± 4.14
Glu	0	65.66 ± 5.26	72.22 ± 5.52	79.44 ± 4.52	328.48 ± 43	361.33 ± 5.46	397.46 ± 8.17
	10	69.08 ± 6.12	75.98 ± 2.28	83.58 ± 2.28	345.58 ± 55	380.14 ± 2.23	418.16 ± 6.52
	20	76.71 ± 4.44	84.38 ± 4.46	92.82 ± 1.51	383.79 ± 64	422.17 ± 5.12	464.39 ± 3.25
	40	80.10 ± 2.55	88.11 ± 3.12	96.92 ± 5.14	400.73 ± 87	440.80 ± 9.55	484.88 ± 5.54
	80	83.37 ± 5.21	91.71 ± 3.55	100.88 ± 1.55	417.11 ± 51	458.82 ± 4.24	504.70 ± 7.21
	160	85.59 ± 2.15	94.15 ± 4.14	103.57 ± 8.13	428.22 ± 85	471.04 ± 6.42	518.14 ± 9.36

*Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

specificity. As from result, it is evident that induction of elicitors was able to induce the accretion of total phenolic and flavonoid contents of *T. resupinatum* ($p < 0.05$), irrespective of method of the culture. The content of phenolic and flavonoid was increased with days of cultivation (Fig. 1), the more influence of elicitor and precursor was seen in flavonoid content. With the increase in the concentration of elicitors and precursors, the content also increased though there was no significant variance seen among them. Gradual increase of content was observed with increasing concentration of MJ, SA, and Glu. There was a slight increase in content with the use of Glu compare to two other molecules. The somatic embryo had more effect on content production than shadow house which was very similar to dark-induced callus. Content of Light-induced callus cultures showed a maximum increase in the content in both phenolic and flavonoid content (Fig. 3). In all the culture status the effect of elicitor and precursor were in following order Glu > SA > MA. The content was comparatively low when the culture was in the dark-induced environment (Fig. 4).

The magnitude of content was varied in following condition light induced > somatic embryo > shadow house > dark-induced. Once when Glu was exposed it showed considerable changes in the manufacture of phenolic and flavonoid compared to MJ and SA-elicited plants culture and non-elicited plant cultures. The outcome showed that the property of MJ, SA, and Glu were effectual on production of phenolic and flavonoid metabolites (Figs. 1–4). The actual phenolic compounds ranged between 22.14 ± 2.31 μg/10 g FW when treated with MA to 42.53 ± 4.21 μg/10 g FW (Fig. 1). Flavonoid content varied from 61.78 ± 3.82 μg/10 g FW to 140.85 ± 5.03 μg/10 g FW (Fig. 1). The results discovered that the highest phenolic and flavonoid content was obtained at highest concentration of Glu after 3 weeks of precursor treatment ($p < 0.05$).

3.2. Determination of DPPH and FRAP antioxidant activity

Elicitor and the precursor used in this study for treatments cultured tissues had a significant effect on the antioxidant activity at the different times averaging 35.95 ± 3.18% to 103.57 ± 8.13% for DPPH assay and 191.76 ± 3.30 to 518.14 ± 9.36 FW (Fresh Weight) for FRAP assay (Tables 1–4). As the week extended the antioxidant activity was grown higher further. The overall results indicate significant effects of treatments in improving the shade-grown plant activity. This activity was not much significant in somatic embryo culture and this was true with all treatments (Tables 1–4). Glu was most effective ($p < 0.05$) and the

Table 4
Determination of effect of MJ, SA and Glu on the DPPH radical scavenging activity and FRAP reducing activity (dark Induced).

Elicitors/precursors	Concentration (μM)	DPPH radical scavenging activity (%)			FRAP assay (μg FeSO4/10 g FW)		
		7th day	14th day	21st day	7th day	14th day	21st day
MJ	0	35.95 ± 3.18	40.05 ± 7.73	44.06 ± 8.43	182.17 ± 8.86	200.39 ± 8.23	220.43 ± 7.30
	10	38.31 ± 4.22	42.14 ± 5.50	46.35 ± 2.25	191.66 ± 3.44	210.82 ± 5.58	231.91 ± 4.45
	20	42.54 ± 8.55	46.80 ± 4.24	51.48 ± 454	212.85 ± 5.28	234.13 ± 7.84	257.55 ± 8.84
	40	44.42 ± 6.44	48.86 ± 2.52	53.75 ± 8.68	222.24 ± 6.52	244.47 ± 4.45	268.91 ± 6.52
	80	46.24 ± 5.36	50.86 ± 5.45	55.95 ± 5.35	231.33 ± 9.65	254.46 ± 5.62	279.91 ± 5.45
	160	47.47 ± 4.63	52.22 ± 2.24	57.44 ± 6.58	237.49 ± 8.37	261.24 ± 8.65	287.36 ± 8.68
SA	0	40.46 ± 7.55	44.50 ± 4.55	48.95 ± 4.84	202.41 ± 6.65	222.66 ± 9.45	244.92 ± 3.86
	10	42.57 ± 2.28	46.82 ± 5.62	51.50 ± 8.45	212.95 ± 5.54	234.25 ± 6.55	257.67 ± 9.59
	20	47.27 ± 8.19	52.00 ± 4.84	57.20 ± 2.56	236.50 ± 9.28	260.15 ± 8.86	286.16 ± 5.45
	40	49.36 ± 6.86	54.29 ± 1.35	59.72 ± 5.49	246.94 ± 7.46	271.63 ± 5.95	298.79 ± 6.28
	80	51.38 ± 545	56.51 ± 5.52	62.16 ± 4.75	257.03 ± 5.55	282.73 ± 7.60	311.01 ± 8.53
	160	52.74 ± 4.28	58.02 ± 2.19	63.82 ± 1.52	263.87 ± 8.88	290.26 ± 4.52	319.29 ± 5.45
Glu	0	50.57 ± 2.54	55.63 ± 5.54	61.19 ± 5.41	253.02 ± 4.12	278.32 ± 6.554	306.15 ± 4.74
	10	53.21 ± 5.45	58.53 ± 3.45	64.38 ± 6.84	266.19 ± 7.24	292.81 ± 8.25	322.09 ± 8.57
	20	59.09 ± 8.72	65.00 ± 5.25	71.50 ± 8.25	295.62 ± 7.55	325.18 ± 5.44	357.70 ± 2.45
	40	61.70 ± 4.54	67.87 ± 4.84	74.65 ± 4.48	308.67 ± 5.48	339.54 ± 7.75	373.49 ± 5.84
	80	64.22 ± 6.52	70.64 ± 4.48	77.71 ± 5.17	321.29 ± 8.42	353.42 ± 4.58	388.76 ± 4.78
	160	65.93 ± 3.14	72.52 ± 8.58	79.77 ± 3.81	329.84 ± 6.54	362.83 ± 6.84	399.11 ± 8.55

*Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

Table 5
Correlation between measured parameters.

Criteria	Parameters	DPPH radical scavenging activity	FRAP reducing activity
Shade house-grown	Total phenolic content	0.86	0.89
	Total flavonoid content	0.82	0.85
	DPPH radical scavenging activity	– –	0.85 –
Somatic embryos	Total phenolic content	0.94	0.98
	Total flavonoid content	0.90	0.93
	DPPH radical scavenging activity	– –	0.94 –
Light induced	Total phenolic content	0.94	0.91
	Total flavonoid content	0.99	0.90
	DPPH radical scavenging activity	– –	0.93 –
Dark Induced	Total phenolic content	0.81	0.82
	Total flavonoid content	0.80	0.81
	DPPH radical scavenging activity	– –	0.80 –

highest antioxidant activity of DPPH and FRAP were recorded at each of its concentration compared to the other two. The correlation between phenolic and flavonoid content was statistically evaluated with antioxidant activity. Results indicate that somatic culture was more productive in terms of antioxidant activity and compare to other shade-grown cultures were less related to the enhancement of metabolites activity. In terms of productivity and activity the following order was observed light induced > somatic > shade house > dark induced (Table 5).

3.3. Determination of enzymatic activity

As both the earlier experiment was evident that the content were more influenced with light-induced callus significantly and so enzymatic evaluation of light-induced callus was done in this study. In this elicitation study, as expected the enzymatic activity was too influenced by all three effectors and precursors, the activity was quite more under the influence of SA than others. Maximum enzyme activity was observed on 21st day when elicited with 160 μM SA. GSH-ST and reductase were more significantly affected and the activity was increased gradually with the concentration of effectors and with the treatment period (Table 6).

4. Discussion

Secondary metabolites, including antibiotics, antioxidants, and many others produced in nature. Plants are unequaled in both complexity and the number of secondary metabolites they create in nature. The ubiquitous phenylpropanoids, flavonoids, and the glucosinolates represent such diverse and large chemically groups (Habig and Jakoby, 1981).

In this study, we produced an evaluation of different culture methods to analyse the level of sustainable metabolite production and antioxidant capacity of T. *resupinatum*. Data analysis showed that all the tested culture methods produced the secondary metabolites in good amount and significantly more when they are exposed to treatment with elicitors and precursors. Among the all approaches, the light-induced callus cultures showed the highest level of phenolic and flavonoid content at each concentration and estimation. Antioxidant activities and enzymatic activities was significantly higher for total phenolic and flavonoid content under influence of Glu treatments when compared to shade-grown and other. For enzymatic production it was seen that in influence of SA had much more effect on production. Pearson's correlation coefficient proves the correlations of various activities of light-induced callus cultures to be significant enough to enhance better than other three. Therefore exogenous application Glu boosts the production of metabolite production which has antioxidant activities in cultures of *T. resupinatum*, signifying that Glu could be activating the enzymatic gene expression of target biosynthetic pathway. These results were in tune with other reports published earlier for *B. candida* (Spollansky et al., 2000), buckwheat (Margna et al., 1985), and *Salvia miltiorrhiza* (Dong et al., 2010), where Glu treatment was significantly efficient than the other elicitors in metabolites production. Here we reported that Glu was superior to MJ and SA in the production of phenolic and flavonoids similar to earlier reports where Phenalanine was the best among others (Reymond and Farmer, 1998) ($p < 0.05$). Amongst the other two, the levels of phenolic and flavonoid contents treated with SA were higher than those for MJ ($p < 0.05$). This was in contrast with those few reports which found that MJ activate the number of defense-related genes

Table 6
Determination of effect of MJ, SA and Glu on enzymatic activity (shade-grown plant).

Elicitors/ precursors	Concentration (µM)	Glutathione peroxidase (µM)			glutathione reductase (µM)			Glutathione S-transferase (GSH-ST)		
		7th day	14th day	21st day	7th day	14th day	21st day	7th day	14th day	21st day
MJ	0	20.49 ± 3.6	24.59 ± 1.6	29.51 ± 3.5	11.91 ± 2.3	13.70 ± 1.6	15.75 ± 2.3	20.05 ± 3.3	23.59 ± 3.3	25.71 ± 3.6
	10	22.36 ± 2.8	26.83 ± 2.2	32.20 ± 2.3	14.15 ± 5.8	16.27 ± 1.8	18.71 ± 1.5	21.06 ± 5.9	24.78 ± 1.5	27.01 ± 2.3
	20	24.23 ± 5.2	29.08 ± 1.5	34.89 ± 1.5	16.39 ± 3.4	18.85 ± 1.2	21.68 ± 3.4	22.07 ± 2.5	25.97 ± 4.4	28.30 ± 4.4
	40	26.10 ± 4.5	31.32 ± 2.4	37.58 ± 5.2	18.63 ± 2.6	21.42 ± 2.5	24.64 ± 1.2	23.08 ± 4.4	27.16 ± 2.6	29.60 ± 2.6
	80	27.97 ± 1.4	33.56 ± 1.53	40.28 ± 1.4	20.87 ± 1.5	24.00 ± 4.4	27.60 ± 4.5	24.09 ± 1.28	28.35 ± 1.8	30.90 ± 4.3
	160	29.84 ± 3.3	35.8.1 ± 3.5	42.97 ± 2.5	23.11 ± 3.8	26.58 ± 2.7	30.56 ± 1.6	25.11 ± 2.7	29.54 ± 4.5	32.19 ± 4.5
SA	0	23.56 ± 5.5	28.2.8 ± 3.9	33.93 ± 4.8	13.70 ± 2.3	15.75 ± 1.2	18.11 ± 2.3	23.06 ± 4.6	27.12 ± 2.2	29.56 ± 3.4
	10	25.71 ± 1.9	30.86 ± 2.8	37.03 ± 3.2	16.27 ± 2.4	18.71 ± 4.4	21.52 ± 1.5	24.22 ± 2.3	28.49 ± 1.4	31.06 ± 2.8
	20	27.86 ± 1.6	33.44 ± 5.6	40.12 ± 2.4	18.85 ± 1.8	21.68 ± 1.3	24.93 ± 2.4	25.38 ± 3.5	29.86 ± 4.7	32.55 ± 1.2
	40	30.02 ± 2.5	36.02 ± 1.5	43.22 ± 3.6	21.42 ± 4.1	24.64 ± 2.5	28.33 ± 1.2	26.54 ± 6.2	31.23 ± 3.6	34.04 ± 4.5
	80	32.17 ± 5.8	38.60 ± 2.7	46.32 ± 4.8	24.00 ± 2.6	27.60 ± 1.6	31.74 ± 3.4	27.71 ± 2.4	32.60 ± 2.3	35.53 ± 2.4
	160	34.32 ± 1.7	41.18 ± 5.4	49.42 ± 2.6	26.58 ± 1.7	30.56 ± 2.6	35.15 ± 1.8	28.87 ± 1.1	33.97 ± 4.5	37.02 ± 1.2
Glu	0	22.54 ± 1.5	27.05 ± 2.5	32.46 ± 1.3	13.10 ± 4.4	15.07 ± 3.3	17.33 ± 2.5	22.05 ± 4.1	25.94 ± 2.9	28.28 ± 4.5
	10	24.60 ± 3.4	29.52 ± 12	35.42 ± 57	15.57 ± .5	17.90 ± 1.5	20.58 ± 3.4	23.17 ± 2.2	27.25 ± 1.6	29.71 ± 2.4
	20	26.65 ± 3.1	31.98 ± 4.1	38.38 ± 4.4	18.03 ± 1.2	20.73 ± 2.4	23.84 ± 2.2	24.28 ± 5.5	28.56 ± 4.5	31.13 ± 3.6
	40	28.71 ± 2.5	34.45 ± 3.4	41.34 ± 1.5	20.49 ± 1.1	23.57 ± 18	27.10 ± 1.5	25.39 ± 4.42	29.87 ± 2.2	32.56 ± 2.3
	80	30.77 ± 1.4	36.92 ± 2.5	44.30 ± 2.1	22.96 ± 1.8	26.40 ± 3.2	30.36 ± 2.6	26.50 ± 1.5	31.18 ± 6.4	33.99 ± 1.5
	160	32.82 ± 4.7	39.39 ± 5.2	47.27 ± 5.2	25.42 ± 2.7	29.23 ± 2.4	33.62 ± 1.4	27.62 ± 3.4	32.49 ± 3.3	35.41 ± 2.4

*Duncan's multiple range test was used to determine a significant difference ($p < 0.05$).

(Kombrink and Somssich, 1997) and thus production seemed to be high in plant culture. In several reports, it was found that 18 defense-related genes were MJ inducible and other 8 were SA-inducible in Arabidopsis (Reymond and Farmer, 1998). Apart from this, it was also noted that MJ was able to activate the intermediate enzymes such as DAHP synthase, flavonoid biosynthesis enzymes, phenylalanine ammonialyase, and tyrosine aminotransferase accountable for the making of phenolic and flavonoid compounds (McConn et al., 1997; Vijayan et al., 1998). Interestingly none of these enzymes expression seems to be altered by SA in Arabidopsis. In this study, we saw that there was a decrease in metabolites dark housed cultures when compared to others and control and so it represents the variations of inducibility in the production secondary metabolites.

In conclusion, this study demonstrates the enhanced metabolites production such as phenolic and flavonoid with increase of antioxidant and enzymatic activities, particularly from *T. resupinatum* in the presence of glutamine as precursor and elicitors.

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Abbreviations

MJ Methyl Jasmonate
SA Salicylic acid
Glu Glutathione
BAP Benzylaminopurine
IAA Indol Acetic Acid
NAA Naphthaleneacetic acid
Kin kinetin
MS Murashige-Skoog
T. resupinatum Trifolium resupinatum
GAE Gallic Acid Equivalents
FW Fresh Weight
GST-ST Glutathione S-Transferase
HPLC High performance liquid chromatography
TBHQ tert-Butylhydroquinone
DPPH Diphenyl-2-picrylhydrazyl
NADPH Nicotinamide adenine dinucleotide phosphate
GSH-ST Glutathione S-Transferase
FRAP Fluorescence recovery after photobleaching

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