Journal of Biotechnology and Bioengineering
Volume 2, Issue 1, 2018, PP 24-30

Effect of Abiotic Stresses on the Adaptation of Metabolites Like Total Phenols, Flavonoids and Alkaloids in Tissue Cultured Plant of *Gloriosa Superba*

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ABSTRACT

To study the effect of stresses on secondary metabolite adaptation, cultured plants of G. superba on Murashige-Skoog (MS) medium were exposed to abiotic stress created artificially by applying different light period (12, 14 and 16 hrs), different pH (6, 7 and 8.5) and different temperature (25, 30 and 35°c). The extract of G. superba, extracted with 95% methanol used to determine the effect of abiotic stress on the quantity of phenol, flavonoids and alkaloids. The content of phenolics was significantly not different in the plants grow under different physical stresses; it ranges from 0.444±0.004 to 1.392±0.02 mg TA/g extract. Plants cultured under different temperature were found to have the highest TPC values 1.176±0.007, 1.301±0.02 mg TA/g extract and 1.392±0.02 mg TA/g extract at 25, 30 and 35˚C temperature respectively. The concentrations of flavonoids in plant extracts ranged from 0.032±0.03 to 0.268±0.002 mg QE/g extract. The highest flavonoid content 0.241±0.002, 0.268±0.002 and 0.247±0.002 mg QE/g extract was identified in the extracts of G. Superba grown under temperature stress in 25, 30 and 35ºC. Alkaloids content was found high (11.308±0.41mg Col/g extract) in plant cultured at a 25ºC temperature, whereas at high temperature (30 and 35ºC) decrease accumulation of alkaloids. Present study concluded that the temperature was highly responsible for the best adaptation of secondary metabolites in G. superba as compared to pH and photoperiods.

Keywords: *Gloriosa superba, Abiotic stress, Alkaloids, Flavonoids, Phenols;*

INTRODUCTION

Stress is an altered physiological condition caused by factors that tend to disrupt the equilibrium strain is any physical and chemical change produced by a stress **[1].** The term stress is used with various meanings, the physiological definition and appropriate term as responses in different situations. Use of elicitors of plant defense mechanisms, i.e. elicitation, has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites [2]**.** Biotic and abiotic stresses which are classified on their origin are used to stimulate the secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations. A wide range of environmental stresses (high and low temperature, drought, alkalinity, salinity, UV stress and pathogen infection) is potentially harmful to the plants. Elicitation has been widely used to increase the production or to induce denovo synthesis of secondary metabolites in invitro plant cell cultures [3]**.** A number of researchers have applied various stresses for enhancement of secondary metabolite production in cultures of the plant cell, tissue and organ. Environmental stresses, such as pathogen attack, UV-irradiation, highlight, wounding, nutrient deficiencies, and temperature and herbicide treatment often increase the accumulation of phenylpropanoids. Higher concentrations of secondary metabolites might result in a more resistant plant. Their production is thought to be costly and reduces plant growth and reproduction [4].

Secondary metabolites, a group of bioactive substances, having diverse classes of organic compounds like alkaloids, terpenoids, phenols, flavonoids, tannins, saponins, etc., are produced through secondary metabolism in different plants. The medicinal value of plants lies in these chemical substances that have definite physiological action on the human body [5]. Phytochemical analysis of ethno medicinal plants

for secondary metabolites is an important area of fundamental research because of its relevance for the discovery of therapeutic agents and providing clues for new sources of bioactive compounds [6]. *G. superba* L., a member of the Liliaceae family is a perennial tuberous climbing herb that distributed in the tropical and subtropical region of India. *G. superba* L. is among some of the modern important medicinal plants, which actually facing local extinction due to climate change. Different parts of the plant have a wide variety of uses, especially in an Indian traditional medicine of the time in immemorial. Tubers and seeds of the *G. superba* L. are an expensive export commodity. Due to the medicinal value, this plant collected from the wild and used as raw material for large-scale medicinal industries, leading to over-exploiting condition, proved to be 95% endangered medicinal plant becomes endangered plant species and included in the red data book [7]. For this reason, the present work is designed to investigate the effect of stresses on the adaptation of secondary metabolites like phenol, flavonoid and alkaloids in the tissue cultured plant of *G. superba*.

The Existing Ranking Methods

For this purpose, cultured plants on Murashige-Skoog (MS) medium were exposed to abiotic stress created artificially by applying different light period (12, 14 and 16 hrs), different pH (6, 7 and 8.5) and different temperature (25, 30 and 35°c). On the medium, in the presence of stressful factors, cultured plants observed for 1 month. After 1 month all plants were harvested to study the effect of abiotic stress on the concentration of secondary metabolites.

Extraction and Phytochemical Analysis

The cultured whole plants were dried at room temperature (39˚C) and made into powder. The dried powder of *G. superba* extracted with 95% methanol (Merck) using soxhlet apparatus. After that, the extract was evaporated in the water bath at 50°C to obtain crude for phytochemical analysis [8].

Determination of Total Phenolic Content

The total phenolic content (TPC) of the crude extracts of *G. superba* plant was determined using the method of Singleton and Rossi [9] with slight modifications. In this method (1:10 v/v diluted with distilled water) Folin Ciocalteau reagent was used for the determination of total phenol. After incubation, development of the blue colour was observed. Finally, absorbance of blue colour in different samples was measured at 725 nm using the spectrophotometer. The phenolic content was calculated as Tannic acid equivalents TA/g extract on the basis of a standard curve of Tannic acid. The results were expressed as tannic acid equivalents TA/g of the plant extract.

Determination Total Flavonoid Content

The total flavonoid content (TFC) of the extract was determined using the aluminium chloride assay through Spectrophotometer [10]. A set of reference standard solutions of quercetin (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) was prepared in the same manner as described earlier. The absorbance for the test and standard solutions were determined against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The TFC was expressed in mg of quercetin equivalents (QE) per gram of extract. All the determinations were carried out three times.

Determination of Total Alkaloids

Total alkaloid content was determined by the spectrophotometric method and studied with the help of colchicine standard curve, as described by John et al., [11]. The absorbance of a red colored complex was measured at 470 nm against the reagent blank. Alkaloid contents were estimated and it was calculated with the help of a standard curve of colchicines. The total alkaloid value is expressed in terms of colchicine equivalent as mg/g of dry weight.

Data Interpretation

The results were statistically analyzed using Excel. The statistical significance result was tested by one-way analysis of variance (ANOVA), at the 0.05 level. The regression analysis was carried out until the least sum of the square was obtained.

Results and Discussion

Effect of Abiotic Stresses on Total Phenol of *G. Superba*

The variation found in the total phenolic content of *G. superba* plant cultured under abiotic stress was shown in Table 1. The content of phenolics was significantly not different in the plants grow under different physical stresses; it ranges from 0.444±0.004 to 1.392±0.02 mg TA/g extract. Plants cultured under different temperature were found to have the highest TPC values 1.176±0.007, 1.301±0.02 mg TA/g extract and

1.392±0.02 mg TA/g extract at 25, 30 and 35˚C temperature respectively. Whereas, plants grow under pH and photoperiod stresses do not show the major difference in the total phenol content of cultured plants. Tannic acid was used as a standard to estimate the concentration of unknown (Figure 1). This value represents that 1g of plant extract contains an amount of phenol equivalent to the pure quantity of tannic acid. Nacif de Abreu and Mazzafera report the effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* choisy. They investigated the effects of water stress and temperature (low and high, constant and alternate) on the phenolic compounds in this species. The results for plants kept in growth chambers indicated that low light intensity might have influenced the levels of the compounds. With the temperature treatments, such an increase was evident only for the phenolic compounds [12]. Similarly Boo *et al.*, 2011 reported the effect of temperature on total polyphenol and anthocyanin contents and the corresponding antioxidant activities. The results showed that at relatively low temperatures, lettuce plants have a high antioxidant and enzymatic status due to the accumulation of phenol [13].

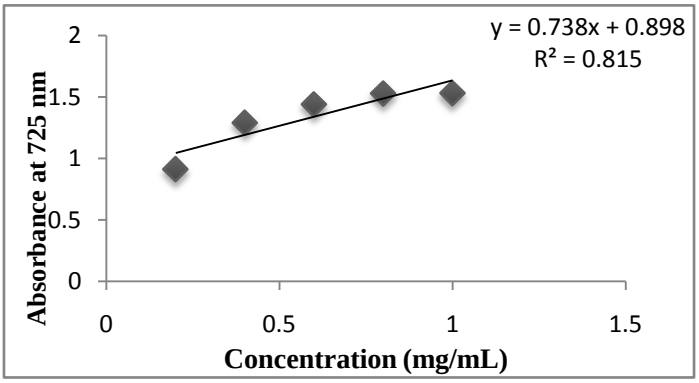

Figure1. *Standard graph of tannic acid for total phenol content assay*

Table1. *Effect of abiotic stress (Temperature, pH and photoperiod) on total phenolic content of G. superba*

Temperature (ºC)	Absorbance	Total phenol (mgTA/g extract)
25	1.863±0.02	1.176±0.007
30	2.059±0.14	1.392±0.02
35	1.976±0.02	1.301±0.02
pH	**Absorbance**	**Total phenol (mgTA/g extract)**
6	1.953±0.028	1.276±0.001
7	1.511±0.007	0.788±0.02
8.5	1.614±0.02	0.901±0.004
Photo period	**Absorbance**	**Total phenol (mgTA/g extract)**
12	1.855±0.02	1.168±0.02
14	1.200±0.028	0.444±0.004
16	1.443±0.02	0.713±0.001

According to ANOVA the results are statistically not significant (P<0.05).

F(obt.)=2.85,F(crt.)=5.14, SS=0.2005, MS=0.0703

Zahir et al., 2014 studied the synergistic effects of drought stress and photoperiods on phenology and secondary metabolism of *Silybum marianum*. They found a positive correlation existed for TPC and TFC when 4 weeks 16/8 h photoperiod treatment was applied. These findings were attributed to the biochemical changes in sprout metabolism, which might influence the production of compounds**.** In the present study, we have evaluated the application of abiotic stress conditions that can induce the production of phenolic content in the *G. superba* [14]. Similarly, Rangel *et al.*, (2014), reported the abiotic stress based bioprocesses for the production of high value antioxidant phenolic compound in plants [15]. Induction of the phenolic acid biosynthesis potentates the survival strategies of plants under various stress conditions [16]**.** It might be due to enlargement of the cellular pool, which induces the scavenging

mechanisms of oxidative injury in plants under adverse environmental conditions [17]. The results agreed with the conclusion of Singha *et al.*, (2014), considered the accumulation of phenolic compounds as the indication of activated defense reaction in the stress resistance of that genotype [18].

Effect of Abiotic Stresses on Total Flavonoid Content of *G. superba*

The summary of the quantities of flavonoids detected in the tested extract is shown in Table 2. The concentration of flavonoids in methanolic extract of *G. superba* was determined using a spectrophotometric method with aluminium nitrate monohydrate [19]. The Quercetine was used as a standard to estimate the concentration of unknown flavonoid (Figure 2). The concentrations of flavonoids in plant extracts ranged from 0.032±0.03 to 0.268±0.002 mg QE/g extract. The highest flavonoid content 0.241±0.002, 0.268±0.002 and 0.247±0.002 mg QE/g extract was identified in the extracts of *G. superba* grown under temperature stress in 25, 30 and 35ºC. Thus flavonoids may have a protective role under stress condition in, similar to proline compound. Flavonoids are frequently induced by abiotic stress and promote roles in plant protection [20]. These compounds accumulated in plant tissue could help to protect themselves from the damaging effects of the act as a free radical scavenger because the hydroxyl groups present in their structure. Moreover, the modifications of flavonoid structure i.e., glycosylation, prenylation and methylation could affect their antioxidant properties, thus they may help inhibit lipid peroxidation in stressed-plants [21]. Our findings indicate that the optimum temperature is responsible for the proper accumulation of flavonoids in plant cells. The highest concentration (0.268±0.002 mg QE/g extract) was observed at 30ºC temperature, which is a normal temperature where the plant can survive easily. Yamamoto *et al.*, (2010), determine the effects temperature on flavonoid concentrations and composition in grape berry skins. They reported that the high temperature significantly reduced the anthocyanin concentrations in the berry skins and modified the anthocyanin composition [22]. The high temperature after variation decreased proanthocyanidin and quercetin concentrations in the berry skins, but the decreased rates were much smaller than that of anthocyanin. Metabolic reactions and growth increase with temperature, although high temperatures may cause cellular damage. High temperatures may also impact the content of bioactive compounds. For example, high temperature leads to a lower content of kaempferol, a type of flavonoid, in the broccoli and the content of lycopene and -carotene in tomato declines significantly at higher temperatures. Pan *et al.*, reported a progressive reduction in anthocyanin content of strawberry fruit at temperatures ranging from 30 to 45°C and they suggested that this explained the onset of oxidative damage [23]. Flavonoid content accumulated under light and pH stress does not show variation. It is similar to the results of Zengqiang *et al.*, [24]. Light intensity is one of the key environmental factors influencing plant growth. The production of total flavonoid content can be explained by the interaction of oxidative stress and photosynthesis. It is widely believed that the synthesis of secondary metabolites in plants is part of the defense responses of plants to oxidative stress. The surplus energy poses an oxidative threat to plant cells. This suggests that young plants were protecting themselves by anthocyanin accumulation and may explain the higher responsiveness of young plants detected in this experiment, even such low radiation intensities as studied Hajiboland *et al.*, [25].

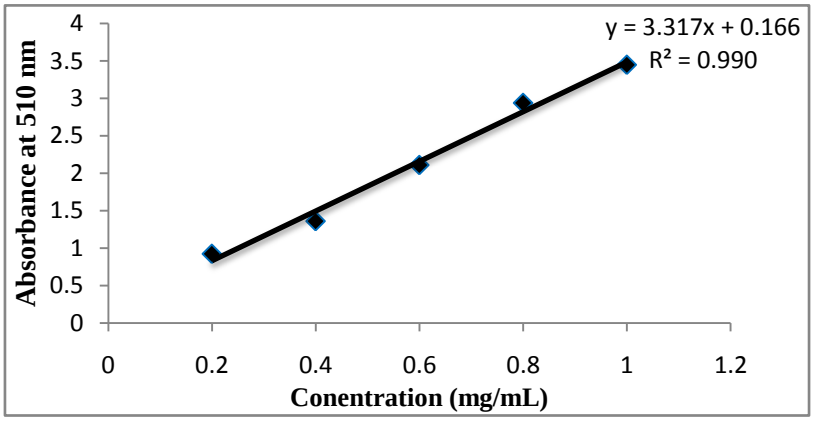

Figure2. *Standard graph of quercetine for total flavonoid content assay*

Table2. *Effect of abiotic stress (Temperature, pH and photoperiod) on total flavonoid content of G. superba*

Temperature (ºC)	Absorbance	Total flavonoid (mg QE/g extract)
25	0.955±0.004	0.241±0.002
30	1.043±0.14	0.268±0.002
35	0.974±0.004	0.247±0.002
pH	**Absorbance**	**Total flavonoid (mg QE/g extract)**
6	0.753±0.028	0.181±0.007
7	0.595±0.02	0.134±0.28
8.5	0.571±0.02	0.128±0.014
Photo period	**Absorbance**	**Total flavonoid (mg QE/g extract)**
12	0.808±0.007	0.198±0.028
14	0.255±0.01	0.032±0.03
16	0.332±0.14	0.055±0.03

According to ANOVA the results are statistically significant (P<0.05).

$F_{2,6}$*(obt.)=6.29,*$F_{2,6}$*(crt.)=5.14, SSB=0.0191, SSW=0.003*

Effect of abiotic stresses on total alkaloid of *G. superba*

The present study was carried out for quantification of alkaloid in the methanolic extract of *G. superba* through the spectrophotometer. Spectrophotometric method is known for its simplicity, sensitivity, and rapid determination. This method is based on the reaction of alkaloid with bromocresol green (BCG), forming a yellow colored product. A similar method was used by John *et al.*, (2014) [11], for estimation of total alkaloids in selected justicia sp**.** The results of total alkaloid were given in Table 3. In this study, colchicine was used as standard (Figure 3). Colchicines and colchicoside are the principal alkaloids of *G. superba*. To study the effect of abiotic stress on the alkaloid accumulation plant grew *in vitro* under stress conditions. In the present research, we selected three different abiotic stresses like temperature, pH and light periods. *In vitro* cultured plants, which were exposed to under temperature stress, shown a significant difference in the concentration of alkaloids as compared to other treatments. Alkaloids content was found high (11.308±0.41mg Col/g extract) in plant cultured at a 25ºC temperature, whereas at high temperature (30 and 35ºC) decrease accumulation of alkaloids. Toivonen *et al.*, (1992), reported the effect of temperature on growth, indole alkaloid accumulation and lipid composition of *Catharanthus roseus* cell suspension cultures [26]**.** Several recent studies have demonstrated that the adaptation of plants to abiotic stresses such as drought, salinity and the temperature is driven by changing in their secondary metabolites. The total alkaloid accumulation in the shoot of *C. roseus* was found increased significantly under drought stress**.**

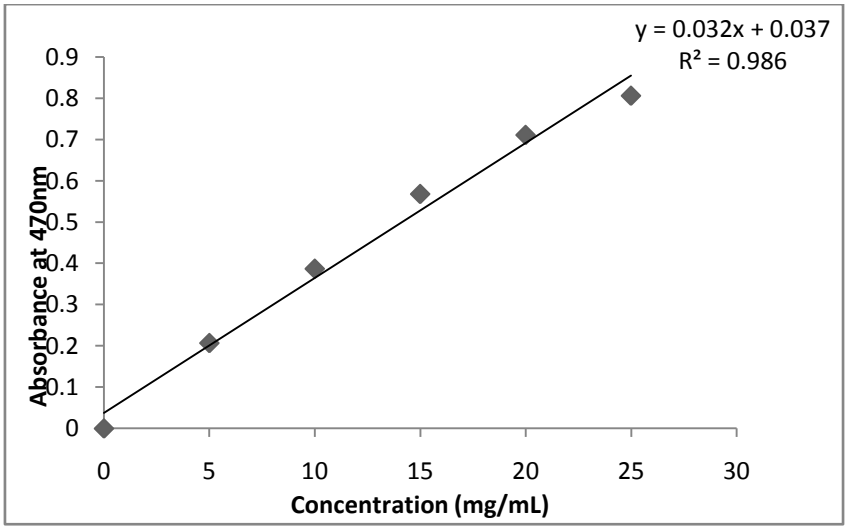

Figure3. *Standard graph of colchicines for total alkaloid content assay*

Table3. *Effect of abiotic stress (Temperature, pH and photoperiod) on total alkaloid content of G. superba*

Temperature (ºC)	Absorbance	Total Alkaloid (mg Col/g extract)
25	0.407±0.028	11.308±0.41
30	0.277±0.004	7.386±0.40
35	0.191±0.007	4.791±0.028
pH	**Absorbance**	**Total Alkaloid (mg Col/g extract)**
6	0.253±0.002	6.662±0.002
7	0.362±0.007	9.950±0.004
8.5	0.186±0.028	4.640±0.01
Photo period	**Absorbance**	**Total Alkaloid (mg Col/g extract)**
12	0.302±0.02	8.140±0.14
14	0.310±0.02	8.381±0.14
16	0.355±0.028	9.739±0.028

According to ANOVA the results are statistically not significant (P<0.05).

$F_{2,6}$(obt.)=0.336,$F_{2,6}$(crt.)=5.14, SSB=2.098, SSW=6.23

The Results Of Photo Periods And Ph Stress Don't Show Major Differences In The Concentration Of Accumulated Alkaloids In *G. Superba*. The Range Of Accumulated Alkaloid Is Between 4.64±0.01 To 9.95±0.004mg Col/G Extract At Various Ph And Light Stresses. The Average Value Of Total Alkaloid Content In *G. Superba* Plant Cultured Under Photoperiodic Stress Is 8.753±0.02 Mg Col/G Extract. Whereas The Average Quantity Of Alkaloid Content Was Found 7.033±0.01mg Col/G Extract At Various Ph Stresses. Mua *Et Al.*, (2009), Reported The Effect Of Abiotic And Biotic Elicitors On Growth And Alkaloid Accumulation Of *Lycoris Chinensis* Seedlings. Alkaloid Concentration In Leaves Increased As Light Period Increased And Peaked At Midday [27].

Conclusion

Present study concluded that abiotic stress factors influence the secondary metabolite production in the plant. The influences are well marked. In fact, productivities depend on the changed stress factor. For example, the influence of temperature, pH and photoperiodic etc. also affect plant adaptation and productivities. The use of *in vitro* plant cell culture for the production of secondary metabolites and pharmaceuticals has made great strides. The use of abiotic stress, changing conditions for secondary metabolite production will provide the basic information for the commercial production of secondary metabolites. The increased level of natural products for medicinal purposes coupled with the low product yields and supply concerns of plant harvest has renewed interest in large-scale plant cell culture technology. Biotic and abiotic factors which influence secondary metabolite production have a bearing on enhancing the potential to over produce useful phytochemicals for varied applications. In this study, temperature shows the best adaptation of secondary metabolites as compare to pH and photo periods in *G. superba* plant. Our study reports the optimization of culture conditions for the production of bioactive secondary metabolite compound from *G. superba*.

Acknowledgement

We wish to thank Principal of Govt. M.V.M, Bhopal for providing research facilities and also thank the Director of CMBT, Bhopal for their valuable guidance and encouragement.

Funding Support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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