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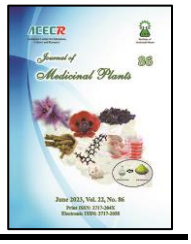

Research Article

Alleviation of low temperatures injury on lemon verbena (*Lippia citriodora* H.B.K.) by exogenous application of adjuvants in anti-chilling formulations

Hanieh Rafiee[1], Ali Mehrafarin[2,3*], Hassanali Naghdi Badi[4], Farahnaz Khalighi-Sigaroodi[2], Esmaiel Jabbari[5], Naleeni Ramawat[6]

[1] *Department of Horticultural Science and Agronomy, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran*

[3] *Medicinal Plants Research Center, Shahed University, Tehran, Iran*

[4] *Department of Agronomy and Plant Breeding, Faculty of Agriculture, Shahed University, Tehran-Qom Express Way, Tehran, Iran; Medicinal Plants Research Center, Shahed University, Tehran, Iran*

[5] *Biomimetic Materials and Tissue Engineering Laboratories, Department of Chemical Engineering, University of South Carolina, Columbia, South Carolina 29208, United States*

[6] *Department of Agronomy, Agriculture University Jodhpur, Rajasthan, India*

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ABSTRACT

Background: Lemon verbena (*Lippia citriodora* H.B.K) from Verbenaceae is a sensitive plant to low temperatures as abiotic stress. **Objective:** The objective of this research was to evaluate the effects of adjuvants and anti-chilling formulations on lemon verbena (*Lippia citriodora* H.B.K) leaves under low temperatures. **Methods:** The combined analysis was done on the basis of a randomized complete blocks design (RCBD) with 28 treatments and 3 replications. The three factors included two anti-chilling formulations (glycerol, and glycerol + polyvinyl alcohol), seven adjuvants formulations (*α*-tocopherol, amino acids of proline + glycine-betaine, and ABA), and two levels of low temperature (5 and 10 °C). **Results:** The treatment of glycerol + proline + glycine-betaine + ABA reduced the damaging effects of low temperature in biomass, essential oil content, and osmoprotectants, while the highest protection by antioxidant pigments was obtained in glycerol + *α*-tocopherol + ABA. Enzymes activities and polyphenol content showed the best results by glycerol + ABA. **Conclusion:** The best formulations were glycerol + proline + glycine-betaine + ABA and glycerol + ABA from the viewpoint of economic yield and also qualitative protection against low temperature, respectively. The efficiency of the mentioned formulations was due to their direct protective function and the indirect influence of adjuvants in a synergistic interaction with other components of the formulation.

Abbreviations: LTs, Low Temperatures; RCBD, Randomized Complete Blocks Design; SOD, Superoxide Dismutase; CAT, Catalase, APX, Ascorbate Peroxidase; GLY, Glycerol; PVA, Polyvinyl Alcohol; PRO, Proline; GB, Glycine-Betaine; EO, Essential oil; αTOC, *α*-Tocopherol; ABA: Abscisic Acid

*Corresponding author: Mehrafarin@imp.ac.ir

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1. Introduction

Lemon verbena (*Lippia citriodora* H.B.K), as a deciduous shrub grows in South America. It belongs to the Verbenaceae family and is cultivated in northern Africa and southern Europe [1]. This plant grows to a height of 1-3 m. It has simple green leaves with short petioles. The plant is woody at the bottom and near the soil surface. The pharmacological properties of lemon verbena are related to the content of phytochemicals in aerial plant parts belonging to phenolic compounds. It is beneficial for digestive disorders due to its anti-inflammatory, analgesic, antipyretic, tonic, and stimulating effects [1]. The leaves with the lemon smell and antispasmodic, sedative, and digestive effects are used for herbal tea [2]. Considering the optimum range of temperature (12-25 °C) for the growth of lemon verbena, low temperature (LT) acts as abiotic stress and limits the production and distribution of this medicinal plant [3]. The antioxidant defense machinery protects plants against the damaging influences of stress. They use these effective antioxidant systems in enzymatic [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX)], and non-enzymatic (e.g. carotenoids and flavonoids) pathways [4]. According to research, the application of anti-chilling eco-friendly formulations like glycerol (GLY) and polyvinyl alcohol (PVA) could improve this defense mechanism against LT damaging effects [5]. However, glycerol or ethylene and propylene glycols and certain surfactants with a high number of ethylene glycol groups, do not or hardly infiltrate the intact cuticle and reveal their effect only on the leaf surface. In addition, glycerol accumulates in *Zygosaccharomyces rouxii* as a stress-tolerant plant by increased retention rather than by increased production during osmotic stress. Glycerol (GLY) is an environmentally friendly, non-toxic, edible, and biodegradable sugar alcohol [6]. Polyvinyl alcohol (PVA) as a synthetic water-soluble polymer is biologically degradable and has excellent chemical resistance and biocompatibility [7].

Adjuvants enhance the efficiency of active ingredients in foliar applications. These important formulation components can be added to a formulation to facilitate the mixing, application, or effectiveness of that compound. Some adjuvants change the formulations to keep them in contact with plant tissues and cover plant surfaces so they do not bead up and roll off. Others increase the formulation's permeation through cuticular wax, cell walls, and/or stomatal openings. Adjuvants help in formulation's effectiveness so that the amount of formulation for achieving the desired effect is sometimes reduced as much as five- or ten-fold [8, 9].

The plant surface and its physicochemical properties influence the responses of plants to foliar application of adjuvants. These substances increase the minimum epidermal conductance, and this effect is possibly related to a decrease in contact angle as well as a change in the cuticular structure [8]. Tocopherols, known as vitamin E, are synthesized in photosynthetic organisms and chloroplasts. These amphiphilic lipid-soluble antioxidants play an important role in protecting plants from singlet oxygen and lipid peroxidation, thus inhibiting the photoinactivation of photosystem II (PSII) and improving low temperature adaptation in plants [10]. Proline (PRO), a molecule involved in the response to plant stresses can act as both a compatible osmolyte and radical scavenger or by its degradation as a supply of energy for regrowth after stressful situations. Moreover, the exogenous application of PRO may also decrease the damaging influences of stresses [11-12].

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Glycine-betaine (GB), an N-trimethyl derivative of glycine, acts as an important osmotic regulator and enhances abiotic stress tolerance in plant tissues. In plants, GB regulates the function of protecting membranes and protein stabilization, and it also maintains cell osmotic pressure. According to research results, GB increased resistance to cold stress damage in Arabidopsis and strawberry plant [13]. ABA as a plant hormone has a regulatory function in plant growth, stomatal closure, and leaf senescence [14]. It has been proven that ABA plays an important role in the development of cold acclimation and ultimately the increase of freezing tolerance in *Betula pendula* Roth. [15]. The aim of this research is to evaluate the effect of different adjuvants in combination with anti-chilling formulations to alleviate the damaging effects of LTs on lemon verbena (*Lippia citriodora* H.B.K).

Materials and Methods

2.1. Plant Materials

Rooted cuttings of *Lippia citriodora* H.B.K. were obtained from the research greenhouse of the Institute of Medicinal Plants, ACECR (35° 54´ N and 50° 53´ E; 1235 m above sea level). Voucher specimen 101 (IMPH) has been deposited in the Institute of Medicinal Plants Herbarium (IMPH). Plants were grown in a greenhouse at day and night temperatures of 20 ± 2 °C and 16 ± 2 °C, with a relative humidity of 55 ± 5 %, an average photosynthetically active radiation of about 650 mmol/m^2.s, and a 16/8 h light/dark cycle. The propagules were planted in plastic pots (0.24 m height × 0.20 m width) filled with 5.5 kg soil. The soil was loam-silt with 0.071 % nitrogen, 48.9 mg/kg phosphorous, 33.6 mg/kg potassium, EC 2.71 /dSm, and pH 8.3.

2.2. Treatment

In the greenhouse, the combined analysis experiment was conducted based on a randomized complete blocks design (RCBD) with 28 treatments and 3 replications in 2019. The first factor was the foliar application of the 2 anti-chilling formulations including glycerol (GLY) and polyvinyl alcohol (PVA). The compound formulations were mentioned in Table 1. The second factor was the foliar application of adjuvants (vitamins, amino acids, and bio-regulators) as auxiliary bioactive compound formulations in seven formulations. The formulations were supplied from Merck KGaA, Darmstadt, Germany (Table 2).

Foliar applications were administered with handheld trigger spray bottles (24 oz. cap.; U.S. Plastic Corp., Lima, OH) until the liquid began to drain off the leaves (50 ml/plant).

The third factor was the LTs of 5 and 10 °C. The foliar application of anti-chilling and adjuvants formulations (Table 3) was done twice in stages of 12 hours before and at the beginning of temperature induction. The mentioned temperatures were induced on completely identical and annual verbena plants with approximately 80 cm in height for 4 days. The temperature treatments were carried out in a Conviron PGV-36 chamber (Controlled Environments Ltd.) with a relative humidity of 70-80 %, a photosynthetic photon flux density of 250 μmol/m^2.s provided by metal halide lamps, and a 12 h photoperiod. After temperature induction, the traits were measured.

2.3. Measurement of photosynthetic and antioxidant pigments

Photosynthetic pigments of chlorophylls *a*, *b*, total chlorophyll, and carotenoid concentrations were determined using a spectrophotometer following the method of Arnon [16]. Anthocyanin concentration was calculated using an extinction coefficient of 33,000 /mol.cm.

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$$\text{Chlorophyll } a \text{ (mg/g FW)} = 12.7(OD663)-2.69(OD645) \times \text{Volume}/1000 \times \text{Weight} \quad (1)$$

$$\text{Chlorophyll } b \text{ (mg/g FW)} = 22.9(OD645)-2.69(OD663) \times \text{Volume}/1000 \times \text{Weight} \quad (2)$$

$$\text{Total chlorophyll (mg/g FW)} = 20.2(OD645) +8.02(OD663) \times \text{Volume}/1000 \times \text{Weight} \quad (3)$$

$$\text{Carotenoids (mg/g FW)} = 100(OD470)-3.27(\text{Chlorophyll } a)-104(\text{Chlorophyll } b)/227 \quad (4)$$

$$\text{Lycopene } 503 = (A \times 537 \times V) / (172 \times 1000 \times V) \quad (5)$$

$$\beta\text{-Carotene } 480 = (A \times 537 \times V) / (139 \times 1000 \times V) \quad (6)$$

Table 1. Treatments of anti-chilling formulations foliar applied on lemon verbena (*Lippia citriodora* H.B.K.)

Abbreviation code	Formulation compounds of anti-chilling polymers		Properties of GLY	Properties of PVA
	GLY (% v/v)	PVA (% v/v)		
G6	6	-	$(HOCH_2)_2$ CHOH 99.5 % purity supplied from Merck KGaA, Darmstadt, Germany	$(C_2H_4O)n$, molar mass of 72000 g/mol viscosity (40 g/L water) of 3.4-4.6 mPas supplied from Merck KGaA, Darmstadt, Germany
G3P3	3	3		

GLY: Glycerol, PVA: Poly vinyl alcohol

Table 2. Treatments of adjuvants foliar applied on lemon verbena (*Lippia citriodora* H.B.K.)

Abbreviation code	Formulation compounds of adjuvants			
	*α*TOC (% v/v)*	Amino acid of Pro (% v/v)	Amino acid of GB (% v/v)	ABA (% v/v)
T0.02	0.02	-	-	-
PG0.5	-	0.5	0.5	-
A0.02	-	-	-	0.02
T0.02PG0.5	0.02	0.5	0.5	-
TA0.02	0.02	-	-	0.02
PG0.5A0.02	-	0.5	0.5	0.02
TA0.02PG0.5	0.02	0.5	0.5	0.02

*α*TOC: *α*-tocopherol; Pro: Proline; GB: Glycine betaine; ABA: Abscisic acid
*($C_{28}H_{48}O_2$) with molecular weight of 416.69 g/mol

Table 3. Treatments of adjuvants in combimation with anti-chilling agents foliar applied on lemon verbena (*Lippia citriodora* H.B.K.)

Row	Abbreviation code	Formulation compounds of adjuvants					
		GLY (% v/v)	PVA (% v/v)	*α*TOC (% v/v)	Pro (% v/v)	GB (% v/v)	ABA (% v/v)
1	G6 + T0.02	6	-	0.02	-	-	-
2	G6 + PG0.5	6	-	-	0.5	0.5	-
3	G6 + A0.02	6	-	-	-	-	0.02
4	G6 + T0.02PG0.5	6	-	0.02	0.5	0.5	-
5	G6 + TA0.02	6	-	0.02	-	-	0.02
6	G6 + PG0.5A0.02	6	-	-	0.5	0.5	0.02
7	G6 + TA0.02PG0.5	6	-	0.02	0.5	0.5	0.02
8	G3P3 + T0.02	3	3	0.02	-	-	-
9	G3P3 + PG0.5	3	3	-	0.5	0.5	-
10	G3P3 + A0.02	3	3	-	-	-	0.02
11	G3P3 + T0.02PG0.5	3	3	0.02	0.5	0.5	-
12	G3P3 + TA0.02	3	3	0.02	-	-	0.02
13	G3P3 + PG0.5A0.02	3	3	-	0.5	0.5	0.02
14	G3P3 + TA0.02PG0.5	3	3	0.02	0.5	0.5	0.02

*α*TOC: *α*-Tocopherol; Pro: Proline; GB: Glycine-betaine; ABA: Abscisic acid

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2.4. Measurement of proline (PRO) and soluble protein

The level of PRO in fresh leaf samples was estimated following the method of Bates et al. [17]. Protein content measurement was performed by the Bradford method [18].

2.5. Determination of polyphenol content

Polyphenol content was determined with the Folin-Ciocalteu reagent according to a procedure described by Singleton and Rossi [19].

2.6. Determination of total soluble solid (TSS)

TSS was estimated by the method as given by Dubois et al (1956) [20].

2.7. Measurement of antioxidant enzymes

For estimating the activities of the antioxidant enzymes, leaf samples (0.5 g) were homogenized in 5 ml of 10 mmol/L phosphate buffer (pH 7.0) containing 4 % (w/v) polyvinylpyrrolidone and 1 mmol/L ethylenediaminetetraacetic acid. The homogenate was centrifuged at 12000 × g for 15 min at 4 °C and the supernatant was used as the source of enzymes for estimation. The extraction was carried out at 4 °C.

The catalase (CAT) activity was determined using the method described by Kato and Shimizu (1987) [21] by monitoring the disappearance of H_2O_2 at 240 nm.

The activity of guaiacol oxidase (POD) was assayed using the method of Asada (1992) [22] by increase in absorbance at 470 nm for 1 min.

APX activity was measured according to Nakano and Asada (1981) by monitoring the decrease in ascorbate oxidation at 290 nm as ascorbic acid was oxidized [23].

SOD (EC 1.15.1.1.) activity was assayed by the nitroblue tetrazolium (NBT) method [24] by 50 % inhibition of NBT reduction under assay conditions.

Hydrogen peroxide (H_2O_2) was determined by following the procedure of Velikova et al. (2000) [25].

2.8. Isolation of the essential oil (EO)

For EO isolation, the harvested leaves were dried 3 days at 40 °C in the oven. Then 50 g of the ground leaves were subjected to hydro-distillation for 3 h using clevenger-type apparatus, according to British Pharmacopoeia [26]. The oils were dried over anhydrous sodium sulfate and kept at 4 °C until analysis. The experiment was repeated three times and the mean was reported as the percentage of EO content on the dry plant.

2.9. Statistical Analysis

The combined analysis was done based on randomized complete blocks design using the SPSS software (ver. 24). Also, Duncan's multiple range tests were used to compare treatment means at a probability level of 0.05.

3. Results

3.1. Effect of LTs

According to Tables (4-6), the LTs significantly affected all measured traits except for POD, H_2O_2, and chlorophyll *a*. The highest amount of leaves, shoot, root, and total dry weight was obtained at 10 °C in comparison with 5 °C. These observations confirm higher content of relative water in leaves at 10 °C. The highest SPAD value, chlorophyll *b*, and total chlorophyll content were attained at 10 °C, while the higher content of total carotenoids, lycopene 503, *β*-carotene, and anthocyanin was related to a lower temperature. Soluble protein, TSS, and PRO content reached the maximum content at 10 °C, while the highest content of polyphenols was observed at the lower temperature. The higher MSI was related to 5 °C. About enzymatic antioxidants, the maximum activity of CAT, APX, and SOD was obtained at 5 °C (Tables 4-6).

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Table 4. Mean comparisons for the effects of different temperatures on morphophysiological traits of lemon verbena (*Lippia citriodora* H.B.K.)

Temp. (°C)	Leaves dry weight /g plant	Stems dry weight /g plant	Shoot dry weight /g plant	Roots dry weight /g plant	Total dry weight /g plant	Relative water content /g plant	SPAD value (SPAD)
5	1.99[b]	6.64[b]	9.83[b]	1.25[b]	10.81[b]	29.20[b]	33.14[b]
10	4.27[a]	12.99[a]	15.97[a]	2.22[a]	18.42[a]	68.45[a]	39.30[a]

*Means in each column followed by the same letter (a-d) are not significantly different according to Duncan's multiple range test at the 5 % level of probability.

Table 5. Mean comparisons for the effects of different temperatures on biochemical traits of lemon verbena (*Lippia citriodora* H.B.K.)

Temp. (°C)	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total Chlorophyll (mg/g FW)	Total Carotenoid (mg/g FW)	Lycopene 503 (mg/g FW)	*β*-carotene (µm/g FW)	Anthocyanin (mg/g FW)
5	0.84	0.54[b]	0.53[b]	2.15[a]	0.09[a]	5.42[a]	1.05[a]
10	0.94	0.79[a]	0.80[a]	1.32[b]	0.06[b]	3.43[b]	0.64[b]

*Means in each column followed by the same letter (a-d) are not significantly different according to Duncan's multiple range test at the 5% level of probability.

Table 6. Mean comparisons for the effects of different temperatures on biochemical traits and enzymatic activities of lemon verbena (*Lippia citriodora* H.B.K.)

Temp. (°C)	Soluble Protein (mg/g FW)	CAT (Units /mg protein)	APX (µmol /mg protein)	POD (Units /mg protein)	H_2O_2 (Units /mg protein)	SOD (µmol /mg protein)	Total soluble solids	Polyphenol content (mg/g DW)	Proline content (mg/g FW)	Membrane stability Index (mg/g FW)
5	12.92[ab]	0.19[a]	0.49[a]	0.08	2.26	219.85[a]	34.25[ab]	7.07[a]	21.92[ab]	94.91[a]
10	15.42[a]	0.09[b]	0.26[b]	0.04	2.36	231.79[ab]	60.02[a]	5.62[ab]	51.630[a]	80.86[b]

*Means in each column followed by the same letter (a-d) are not significantly different according to Duncan's multiple range test at the 5 % level of probability.

3.2. Effect of anti-chilling formulations

According to the results of variance analysis, the effect of anti-chilling formulations was significant on biomass as well as biochemical traits except for leaves dry weight, SPAD value, lycopene 503, *β*-carotene, and soluble protein. As shown in Table 7, the number of stems, shoots, roots, and total dry weight was increased by foliar application of G6. The highest relative water content (RWC) in leaves was reached in plants treated by G6. The content of chlorophylls (*a*, *b*, total chlorophyll), total carotenoids, and anthocyanins were elevated by the treatment of G6. Activities of CAT, APX, POD enzymes were increased by foliar application of G6, while the maximum activity of SOD enzyme was observed in plants foliar applied by G3P3. The Polyphenol content and MSI increased by treatment of G3P3. The content of TSS and PRO was raised by foliar the application of G6 (Tables 8 and 9).

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Table 7. Mean comparisons for the effects of anti-chilling formulations on morphophysiological traits of lemon verbena (*Lippia citriodora* H.B.K.)

Anti-chilling agents*	Leaves dry weight /g plant	Stems dry weight /g plant	Shoot dry weight /g plant	Root dry weight /g plant	Total dry weight /g plant	Relative water content /g plant	SPAD value (SPAD)
G6	3.99	12.76[a]	16.12[a]	2.25[a]	18.71[a]	57.97[a]	37.49
G3P3	2.28	6.88[b]	9.69[b]	1.22[b]	10.52[b]	39.69[b]	34.96

*G6: 6 % v/v Glycerol, G3P3: 3 % v/v Glycerol + 3 % v/v Polyvinyl alcohol

Table 8. Mean comparisons for the effects of anti-chilling formulations on biochemical traits of lemon verbena (*Lippia citriodora* H.B.K.)

Anti-chilling agent*	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total Chlorophyll (mg/g FW)	Total Carotenoid (mg/g FW)	Lycopene 503 (mg/g FW)	*β*-carotene (µm/g FW)	Anthocyanin (mg/g FW)
G6	0.92[a]	0.70[a]	0.73[a]	1.99[a]	0.08	4.99	1.01[a]
G3P3	0.86[b]	0.63[b]	0.60[b]	1.48[b]	0.07	3.86	0.68[b]

*G6: 6 % v/v Glycerol, E6: 6 % v/v Ethylene glycol, G3P3: 3 % v/v Glycerol + 3 % v/v Polyvinyl alcohol

Table 9. Mean comparisons for the effects of anti-chilling formulations on biochemical traits and enzymatic traits of lemon verbena (*Lippia citriodora* H.B.K.)

Anti-chilling agent*	Soluble Protein (mg/g FW)	CAT (Units /mg protein)	APX (µmol /mg protein)	POD (Units /mg protein)	H_2O_2 (Units /mg protein)	SOD (µmol /mg protein)	Total soluble solids	Polyphenol content (mg/g DW)	Proline content (mg/g FW)	Membrane stability Index (mg/g FW)
G6	15.39	0.18[a]	0.50[a]	0.09[a]	2.26[b]	199.45[b]	51.93[a]	4.99[b]	48.98[a]	85.51[b]
G3P3	12.95	0.10[b]	0.25[b]	0.04[b]	2.35[a]	251.24[a]	42.34[b]	7.69[a]	24.57[ab]	90.26[a]

*G6: 6 % v/v Glycerol, E6: 6 % v/v Ethylene glycol, G3P3: 3 %v/v Glycerol + 3 % v/v Polyvinyl alcohol

3.3. Effect of adjuvants (auxiliary bioactive compounds)

Based on variance analysis results, the effect of adjuvants was significant on measured biomass and biochemical traits except for roots dry weight, chlorophyll *a*, total chlorophyll, lycopene 503, and *β*-carotene. As the results of mean comparisons showed, the greatest amount of morphological traits was attained in plants treated by PG0.5A0.02, while the lowest of those was observed by foliar application of T0.02. The maximum and minimum RWC was related to PG0.5A0.02 and TA0.02, respectively. The plants treated by TA0.02PG0.5 showed the highest SPAD value, but the foliar application of PG0.5 caused the least value of that. The content of chlorophyll *b*, total carotenoids, and anthocyanin was elevated by foliar application of TA0.02, while the lowest content of mentioned traits was related to treatment of T0.02. The content of soluble protein, TSS, and PRO was increased by the application of PG0.5A0.02. Conversely, the lowest of those was attained by foliar application of TA0.02 and T0.02 without significant differences. Polyphenol content and MSI similarly showed the highest and the lowest level by treatment of PG0.5 and A0.02, respectively. The best treatment in the activity of the enzymatic antioxidants (APX and POD) was A0.02, while the highest activity of CAT and SOD enzymes was obtained by TA0.02, and TA0.02PG0.5, respectively. The lowest activity of H_2O_2 was observed by the application of TA0.02 (Tables 10-12).

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Table 10. Mean comparisons for the effects of adjuvants on morphophysiological traits of lemon verbena (*Lippia citriodora* H.B.K.)

Adjuvant*	Leaves dry weight /g plant	Stems dry weight /g plant	Shoot dry weight /g plant	Root dry weight /g plant	Total dry weight /g plant	Relative water content (%)	SPAD value (SPAD)
T0.02	2.25^{d}	7.60^{c}	8.89^{d}	1.53	10.72^{c}	44.11^{cd}	34.14^{ab}
PG0.5	3.51^{b}	11.42^{ab}	15.81^{a}	1.67	16.27^{a}	47.65^{bc}	33.25^{b}
A0.02	2.65^{c}	8.67^{c}	12.28^{c}	1.97	12.40^{b}	45.47^{cd}	37.43^{a}
T0.02PG0.5	2.87^{c}	10.17^{b}	12.91b^{c}	1.81	15.77^{a}	45.03^{cd}	35.57^{ab}
TA0.02	2.85^{c}	7.85^{c}	10.29^{d}	1.50	13.32^{b}	42.10^{d}	38.10^{a}
PG0.5A0.02	4.12^{a}	12.44^{a}	15.68^{a}	2.05	17.40^{a}	65.97^{a}	36.88^{ab}
TA0.02PG0.5	3.69^{b}	10.58^{b}	14.44^{ab}	1.63	16.44^{a}	51.46^{b}	38.19^{a}

*T0.02: 0.02 % v/v α-tocopherol, PG0.5: 0.5 % v/v Proline + 0.5 % v/v Glycine betaine, A0.02: 0.02 % v/v Abscisic acid, T0.02PG0.5: 0.02 % α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine, TA0.02: 0.02 % v/v α-tocopherol + 0.02 %v/v Abscisic acid, PG0.5A0.02: 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid, TA0.02PG0.5: 0.02 % v/v α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid.

Table 11. Mean comparisons for the effects of adjuvants on biochemical traits of lemon verbena (*Lippia citriodora* H.B.K.)

Adjuvant*	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total chlorophyll (mg/g FW)	Total carotenoid (mg/g FW)	Lycopene 503 (mg/g FW)	*β*-carotene (µm/g FW)	Anthocyanin (mg/g FW)
T0.02	0.89	0.61^{ab}	0.64	1.52^{d}	0.08	4.39	0.75^{d}
PG0.5	0.89	0.59^{ab}	0.65	1.72^{bc}	0.07	4.38	0.80^{cd}
A0.02	0.89	0.70^{a}	0.70	1.75^{b}	0.08	4.59	0.86^{b}
T0.02PG0.5	0.90	0.69^{a}	0.68	1.63^{cd}	0.08	4.60	0.82^{bc}
TA0.02	0.91	0.72^{a}	0.70	1.97^{a}	0.08	4.57	1.05^{a}
PG0.5A0.02	0.88	0.64^{ab}	0.64	1.57^{d}	0.08	4.06	0.78^{cd}
TA0.02PG0.5	0.89	0.71^{a}	0.63	1.97^{a}	0.08	4.40	0.85^{b}

*T0.02: 0.02 % v/v α-tocopherol, PG0.5: 0.5 % v/v Proline + 0.5 % v/v Glycine betaine, A0.02: 0.02 % v/v Abscisic acid, T0.02PG0.5: 0.02 % α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine, TA0.02: 0.02 % v/v α-tocopherol + 0.02 % v/v Abscisic acid, PG0.5A0.02: 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid, TA0.02PG0.5: 0.02 % v/v α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid.

Table 12. Mean comparisons for the effects of adjuvants on biochemical traits and enzymatic activities of lemon verbena (*Lippia citriodora* H.B.K.)

Adjuvant*	Soluble Protein (mg/g FW)	CAT (Units /mg protein)	APX (µmol /mg protein)	POD (Units /mg protein)	H_2O_2 (Units /mg protein)	SOD (µmol /mg protein)	Total soluble solids (mg/g DW)	Polyphenol content (mg/g DW)	Proline content (mg/g FW)	Membrane stability Index (mg/g FW)
T0.02	13.66^{bc}	0.11^{c}	0.23^{d}	0.06^{c}	2.37^{b}	210.76^{e}	42.20^{d}	6.02^{c}	22.08^{e}	87.78^{b}
PG0.5	14.48^{ab}	0.11^{c}	0.37^{b}	0.06^{cd}	2.66^{a}	230.83^{c}	45.47^{c}	7.01^{a}	37.53^{cd}	94.80^{a}
A0.02	13.92^{bc}	0.16^{a}	0.44^{a}	0.10^{a}	2.87^{a}	226.51^{c}	49.03^{b}	5.29^{d}	35.58^{d}	85.45^{b}
T0.02PG0.5	14.54^{ab}	0.13^{b}	0.32^{c}	0.04^{e}	2.14^{cd}	212.52^{e}	46.26^{c}	6.65^{ab}	39.37^{c}	87.18^{b}
TA0.02	12.62^{c}	0.16^{a}	0.44^{a}	0.073^{b}	1.97^{d}	220.01^{d}	44.67^{cd}	6.55^{b}	20.74^{e}	86.02^{b}
PG0.5A0.02	15.39^{a}	0.16^{a}	0.42^{ab}	0.06^{cd}	1.91^{d}	236.39^{b}	53.41^{a}	6.15^{c}	53.57^{a}	85.49^{b}
TA0.02PG0.5	14.59^{ab}	0.13^{b}	0.41^{a}	0.05^{de}	2.26^{bc}	243.72^{a}	48.93^{b}	6.70^{ab}	48.57^{b}	88.47^{ab}

*T0.02: 0.02 % v/v α-tocopherol, PG0.5: 0.5 %v/v Proline + 0.5 %v/v Glycine betaine, A0.02: 0.02 %v/v Abscisic acid, T0.02PG0.5: 0.02 % α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine, TA0.02: 0.02 % v/v α-tocopherol + 0.02 % v/v Abscisic acid, PG0.5A0.02: 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid, TA0.02PG0.5: 0.02 % v/v α-tocopherol + 0.5 % v/v Proline + 0.5 % v/v Glycine betaine + 0.02 % v/v Abscisic acid.

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3.4. Effect of adjuvants in anti-chilling formulations under LTs

The interaction effect of two LTs, anti-chilling formulations, and adjuvants was significant on all biomass traits. However, in biochemical traits, it was not significant on chlorophylls *a*, *b*, total chlorophyll, lycopene 503, *β*-carotene, and POD enzyme activity. According to mean comparisons, the application of G6 in both temperatures increased the dry weight of biomass in comparison with G3P3. Although, the greatest rate of biochemical traits was observed at 5 °C in comparison with 10 °C. The effect of G6 in combination with PRO, GB, and ABA (treatment 6) caused the highest amount of morphological parameters, while the least of those was observed in treatment 8 (Table 13). The relative water content was elevated by application of treatment 6 and it reached the lowest level by foliar application of treatment 12. The greatest and the least SPAD value was related to treatments 5 and 11, respectively (Table 13). As it is shown in Table 14, the maximum content of total carotenoids was observed in plants treated with GLY in combination with *α*TOC and ABA, while the minimum of that was obtained by treatments 12, 13, and 14. The anthocyanin content was raised by treatment 5 and it decreased by treatment 8 in both temperatures. The highest content of soluble protein was related to treatment 6 and the least to treatment 8 (Table 15). The higher rate of enzyme activities was attributed to 5 °C. The maximum activity of the enzyme CAT was related to plants foliar applied by treatment 3. The lowest activity of CAT was attained by the application of G3P3. The application of GLY in combination with ABA resulted in higher activity of the APX enzyme. The lowest activity and amount of H_2O_2 was related to treatment 3 while treatment 8 caused the highest amount of that. The maximum activity of SOD was achieved by foliar application of treatments 3 and 5 and the minimum of that was observed by 8 (Table 15). The higher rate of osmolytes was observed at 10 °C. The foliar application of treatment 6 resulted in the greatest content of total soluble solids in both LTs. However, the lowest of that was obtained by treatment 8 (Table 14). The content of PRO was raised by foliar application of treatments 6 and 7 without significant differences in both temperatures. The best treatments in decreasing polyphenol content in foliar-applied plants were 3 and 5 without significant differences and the least effect was obtained by treatment 8. Treatment 3 caused the best and the lowest MSI in both LTs, while the highest of that was observed in treatment 8 (Table 14).

As shown in Fig. 1, the best treatment in increasing the essential oil content was the combination of G6 with PRO, GB, ABA (treatment 6), and the least of that was obtained by treatment 8 at both LTs.

Table 13. Mean comparisons for the effects of adjuvants and anti-chilling formulations on morphophysiological traits of lemon verbena (*Lippia citriodora* H.B.K.)

Formulation*	Temp. (°C)	Leaves dry weight /g plant	Stems dry weight /g plant	Shoot dry weight /g plant	Root dry weight /g plant	Total dry weight /g plant	Relative water content (%)	SPAD (SPAD Value)
1	5	1.98 ± 0.69	5.77 ± 1.46	10.47 ± 2.02	1.41 ± 0.46	12.14 ± 1.68	25.00 ± 5.66	33.45 ± 10.78
	10	4.12 ± 1.46	9.59 ± 2.27	13.81 ± 2.54	2.39 ± 0.62	14.79 ± 1.86	72.77 ± 12.21	31.92 ± 10.80
2	5	2.7 ± 0.95	8.71 ± 2.32	12.64 ± 2.16	1.80 ± 0.37	14.41 ± 2.49	38.23 ± 8.29	33.1 ± 11.44
	10	6.43 ± 1.15	19.94 ± 3.67	23.77 ± 3.96	2.82 ± 0.42	26.76 ± 3.40	78.27 ± 12.71	34.65 ± 11.66
3	5	1.91 ± 0.67	8.10 ± 2.02	11.62 ± 2.19	1.62 ± 0.53	13.06 ± 1.04	29.98 ± 8.60	35.5 ± 11.80
	10	4.75 ± 1.69	13.68 ± 1.92	17.60 ± 5.15	2.82 ± 0.55	19.30 ± 1.05	71.73 ± 10.14	42.55 ± 19.11

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Table 13. Mean comparisons for the effects of adjuvants and anti-chilling formulations on morphophysiological traits of lemon verbena (*Lippia citriodora* H.B.K.) (Continued)

Formulation *	Temp. (°C)	Leaves dry weight	Formulation *	Temp. (°C)	Leaves dry weight	Formulation *	Temp. (°C)	Leaves dry weight
4	5	2.59 ± 0.91	8.43 ± 2.03	11.27 ± 3.25	1.67 ± 0.81	14.66 ± 2.66	34.43 ± 5.39	35.3 ± 12.11
	10	4.73 ± 1.09	19.35 ± 4.02	23.19 ± 7.62	2.74 ± 0.80	26.93 ± 2.55	74.49 ± 14.29	40.9 ± 14.53
5	5	1.99 ± 0.70	6.03 ± 2.31	10.06 ± 1.33	1.71 ± 0.55	13.02 ± 1.83	25.11 ± 8.28	36.75 ± 12.41
	10	4.49 ± 1.59	13.53 ± 3.11	15.66 ± 2.69	2.61 ± 0.96	18.27 ± 1.86	70.80 ± 13.81	44.1 ± 19.33
6	5	3.19 ± 1.13	13.26 ± 22.60	14.34 ± 5.65	1.94 ± 0.43	16.05 ± 5.72	75.35 ± 5.60	35.1 ± 11.72
	10	7.24 ± 1.42	22.61 ± 4.58	29.04 ± 6.72	3.74 ± 0.55	31.78 ± 8.10	96.34 ± 16.56	41.9 ± 12.29
7	5	2.95 ± 1.05	9.30 ± 2.95	13.41 ± 2.02	2.05 ± 0.51	15.21 ± 3.55	36.73 ± 7.56	36.4 ± 12.68
	10	6.82 ± 1.83	20.4 ± 7.89	26.58 ± 6.33	2.99 ± 0.87	29.40 ± 7.84	82.29± 14.72	43.2 ± 19.31
8	5	1.30 ± 0.46	5.44 ± 1.27	8.39 ± 0.99	0.62 ± 0.23	9.01 ± 1.23	17.37 ± 4.94	33.45 ± 12.65
	10	1.62 ± 0.61	9.60 ± 1.41	6.63 ± 1.69	1.83 ± 0.64	6.94 ± 1.59	61.31 ± 9.99	37.75 ± 13.47
9	5	1.76 ± 0.62	5.91 ± 2.14	13.80 ± 3.39	0.51 ± 0.23	9.24 ± 2.86	24.32 ± 3.26	29.55 ± 8.85
	10	3.13 ± 0.50	11.13 ± 2.41	13.04 ±2.34	1.62 ± 0.60	10.81 ± 1.75	49.78± 9.32	35.7 ± 12.36
10	5	1.14 ± 0.40	3.85 ± 1.40	7.17 ± 2.11	0.83 ± 0.27	6.71 ± 1.75	20.75 ± 5.30	31.5 ± 9.25
	10	2.79 ± 0.99	9.06 ± 1.96	12.73 ± 2.77	1.70 ± 0.78	12.77 ± 2.27	59.42 ± 14.24	40.5 ± 18.24
11	5	1.42 ± 0.62	4.89 ± 1.32	6.19 ± 1.24	0.8 ± 0.24	6.90 ± 1.49	21.88 ± 2.73	25.13 ± 4.97
	10	2.78 ± 0.50	8.01 ± 2.09	11 ± 2.10	2.08 ± 0.85	16.02± 3.69	49.31 ± 14.49	40.95 ± 17.52
12	5	1.09 ± 0.38	5.00 ± 1.82	8.35 ± 2.23	1.22 ± 0.48	9.60 ± 1.67	16.94 ± 3.37	33.03 ± 10.21
	10	3.83 ± 1.35	6.86 ± 1.50	11.19 ± 2.74	1.25 ± 0.33	12.4 ± 3.24	55.54 ± 12.25	38.5 ± 13.81
13	5	1.97 ± 0.69	3.98 ± 1.84	6.62 ± 2.11	0.75 ± 0.23	7.29 ± 2.28	22.23 ± 9.10	33.38 ± 13.67
	10	4.08 ± 1.45	9.94 ± 1.94	12.74 ± 1.78	1.77 ± 0.74	14.46 ± 2.38	69.96 ± 13.96	37.8 ± 16.28
14	5	1.91 ± 0.48	4.34 ± 1.30	7.41 ± 1.85	0.73 ± 0.32	7.95 ± 1.55	20.53 ± 4.76	33 ± 11.66
	10	3.08 ± 1.09	8.29 ± 1.99	10.37 ± 2.29	0.75 ± 0.25	13.19 ± 1.29	66.27 ± 19.75	40.15 ± 14.22

*1: G6 + T0.02; 2: G6 + PG0.5; 3: G6 + A0.02; 4: G6 + T0.02PG0.5; 5: G6 + TA0.02; 6; G6 + PG0.5A0.02; 7: G6 + TA0.02PG0.5; 8: G3P3 + T0.02; 9: G3P3 + PG0.5; 10: G3P3 + A0.02; 11: G3P3 + T0.02PG0.5; 12: G3P3 + TA0.02; 13: G3P3 + PG0.5A0.02; 14: G3P3 + TA0.02PG0.5

Table 14. Mean comparisons for the effects of adjuvants and anti-chilling formulations on biochemical traits of lemon verbena (*Lippia citriodora* H.B.K.)

Formulation*	Temp. (°C)	Total Carotenoid (mg/g FW)	Anthocyanin (mg/g FW)	Total soluble solids (mg/g DW)	Polyphenols (mg/g DW)	Membrane stability index (mg/g DW)	Proline (mg/g DW)
1	5	2.17 ± 0.22	1.16 ± 0.04	35.33 ± 6.84	6.57 ± 1.62	96.86 ± 3.41	17.94 ± 4.62
	10	1.47 ± 0.28	0.64 ± 0.08	63.68 ± 11.33	5.25 ±0.26	81.95 ± 13.25	52.36 ± 4.39
2	5	2.06 ± 0.10	1.02 ± 0.12	40.75 ± 8.70	5.87 ± 1.37	93.89 ± 3.49	35.06 ± 5.34
	10	1.39 ± 0.14	0.66 ± 0.12	70.35 ± 9.17	5.09 ± 1.09	78.71 ± 8.10	73.34 ± 11.84
3	5	2.61 ± 0.32	1.33 ± 0.31	37.19 ± 7.47	4.93 ± 1.04	86.99 ± 12.19	19.94 ± 0.69
	10	1.55 ± 0.20	0.77 ± 0.15	69.72 ± 12.85	3.40 ± 0.49	96.69 ± 25.80	61.56 ± 7.66
4	5	2.18 ± 0.26	1.15 ± 0.11	37.19 ± 7.47	5.71 ± 1.31	94.11 ± 4.88	23.38 ± 8.30
	10	1.42 ± 0.38	0.75 ± 0.15	70.18 ± 13.51	4.23 ± 0.08	80.35 ± 12.69	68.30 ± 10.05
5	5	3.42 ± 0.34	1.75 ± 0.12	35.39 ± 6.86	4.94 ± 1.04	89.56 ± 10.20	16.84 ± 1.12
	10	1.71 ± 0.37	0.94 ± 0.01	66.79 ± 12.37	3.60 ± 0.56	71.52 ± 6.09	55.63 ± 6.29
6	5	2.39 ± 0.20	1.31 ± 0.08	49.22 ± 11.65	5.33 ± 1.18	87.77 ± 3.26	58.69 ± 6.64
	10	1.48 ± 0.41	0.71 ± 0.06	71.14 ± 9.39	3.72 ± 0.61	71.97 ± 2.28	82.68 ± 8.05
7	5	3.24 ± 0.41	1.32 ± 0.34	45.67 ± 6.22	5.54 ± 0.16	92.85 ± 6.88	55.68 ± 12.67
	10	1.59 ±0.26	0.83 ± 0.01	69.61 ± 8.67	3.76 ± 0.62	75.19 ± 7.12	82.02 ± 8.56
8	5	1.80 ± 0.19	0.66 ± 0.09	21.95 ± 2.67	9.23 ± 0.43	101 ± 1.67	3.25 ± 0.81
	10	1.18 ± 0.21	0.55 ± 0.13	42.28 ± 9.23	8.70 ± 0.96	95.34 ± 3.66	11.48 ± 1.98
9	5	1.96 ± 0.08	0.91 ± 0.11	30.59 ± 5.25	9.24 ± 1.14	100.65 ± 3.30	13.57 ± 1.26
	10	0.96 ± 0.29	0.59 ± 0.15	56.06 ± 8.84	8.27 ± 0.80	87.64 ± 9.09	49.12 ± 10.34
10	5	1.86 ± 0.11	0.75 ± 0.20	26.07 ± 3.81	7.53 ± 1.25	95.58 ± 4.35	9.82 ± 0.64
	10	1.14 ± 0.37	0.67 ± 0.11	49.85 ± 6.94	5.52 ± 0.34	81.77 ± 14.36	28.73 ± 3.10

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Table 14. Mean comparisons for the effects of adjuvants and anti-chilling formulations on biochemical traits of lemon verbena (*Lippia citriodora* H.B.K.) (Continued)

Formulation*	Temp. (°C)	Total Carotenoid	Formulation*	Temp. (°C)	Total Carotenoid	Formulation*	Temp. (°C)
11	5	1.92 ± 0.42	1.02 ± 0.10	28.16 ± 4.46	8.98 ± 0.34	96.91 ± 7.55	13.01 ± 1.17
	10	1.15 ± 0.14	0.43 ± 0.15	51.90 ± 0.66	7.45 ± 0.905	87.25 ± 10.97	37.93 ± 6.37
12	5	1.55 ± 0.15	0.91 ± 0.006	27.37 ± 4.21	7.56 ± 1.26	97.18 ± 8.20	6.58 ± 0.91
	10	1.18 ± 0.44	0.66 ± 0.08	43.92 ± 5.35	6.13 ± 0.75	82.36 ± 8.29	18.87 ± 0.34
13	5	1.50 ± 0.35	0.69 ± 0.09	33.02 ± 6.05	8.59 ± 0.91	97.45 ± 6.63	17.30 ± 4.72
	10	1.17 ± 0.20	0.44 ± 0.09	57.92 ± 9.44	6.67 ± 1.66	82.22 ± 6.84	50.99 ± 11.01
14	5	1.64 ± 0.16	0.90 ± 0.08	31.66 ± 4.04	8.90 ± 1.03	97.87 ± 5.61	16.84 ± 1.12
	10	1.22 ± 0.08	0.41 ± 0.07	56.87 ± 2.42	6.81 ± 0.99	86.00 ± 8.60	49.98 ± 3.55

*1: G6 + T0.02; 2: G6 + PG0.5; 3: G6 + A0.02; 4: G6 + T0.02PG0.5; 5: G6 + TA0.02; 6; G6 + PG0.5A0.02; 7: G6 + TA0.02PG0.5; 8: G3P3 + T0.02; 9: G3P3 + PG0.5; 10: G3P3 + A0.02; 11: G3P3 + T0.02PG0.5; 12: G3P3 + TA0.02; 13: G3P3 + PG0.5A0.02; 14: G3P3 + TA0.02PG0.5

Table 15. Mean comparisons for the effects of adjuvants and anti-chilling formulations on biochemical traits and enzymatic activities of lemon verbena (*Lippia citriodora* H.B.K.)

Formulation*	Temp. (°C)	Soluble protein (mg/g FW)	CAT (Units /mg Protein)	SOD (µmol/mg Protein)	APX (µmol/mg Protein)	H_2O_2 (Units /mg Protein)
1	5	12.96 ± 2.31	0.10 ± 0.02	215.88 ± 9.86	0.34 ± 0.04	3.81 ± 0.71
	10	14.99 ± 3.84	0.09 ± 0.01	242.37 ± 6.39	0.31 ± 0.02	3.27 ± 0.19
2	5	14.51 ± 3.18	0.16 ± 0.02	223.60 ± 3.18	0.59 ± 0.05	2.32 ± 0.14
	10	16.73 ± 3.60	0.09 ± 0.01	253.99 ± 11.52	0.42 ± 0.03	2.36 ± 0.21
3	5	13.41 ± 2.14	0.34 ± 0.02	290.19 ± 35.67	0.63 ± 0.17	2.21 ± 0.10
	10	16.34 ± 2.90	0.12 ± 0.01	287.24 ± 13.66	0.60 ± 0.02	2.37 ± 0.40
4	5	14.34 ± 4.15	0.24 ± 0.04	236.08 ± 3.18	0.58 ± 0.14	2.44 ± 0.25
	10	16.64 ± 2.47	0.10 ± 0.01	254.80 ± 8.27	0.42 ± 0.04	2.55 ± 1.02
5	5	13.21 ± 3.24	0.34 ± 0.03	284.12 ± 8.11	0.62 ± 0.05	3.05 ± 0.50
	10	14.78 ±3.77	0.12 ± 0.01	279.76 ± 8.27	0.60 ± 0.02	3.00 ± 0.06
6	5	16.51 ± 3.91	0.34 ± 0.02	261.04 ± 8.27	0.60 ± 0.05	2.62 ± 0.25
	10	17.74 ± 2.51	0.11 ± 0.01	265.39 ± 6.86	0.42 ± 0.07	2.88 ± 0.33
7	5	16.19 ± 2.47	0.26 ± 0.04	248.56 ± 8.27	0.59 ± 0.26	2.59 ± 0.11
	10	17.16 ± 3.14	0.11 ± 0.00	258.58 ± 10.52	0.42 ± 0.07	2.61 ± 0.18
8	5	9.29 ± 2.43	0.18 ± 0.01	169.02 ± 5.49	0.20 ± 0.04	1.60 ± 0.16
	10	13.75 ± 2.65	0.05 ± 0.02	167.44 ± 3.43	0.17 ± 0.06	1.40 ± 0.04
9	5	12.57 ± 2.83	0.17 ± 0.01	173.68 ± 3.18	0.22 ± 0.05	2.03 ± 0.17
	10	14.99 ± 3.84	0.04 ± 0.01	179.92 ± 8.27	0.2 ± 0.01	2.40 ± 0.37
10	5	10.74 ± 2.92	0.11 ± 0.01	211.12 ± 13.66	0.32 ± 0.05	1.60 ± 0.23
	10	15.21 ± 3.41	0.05 ± 0.01	229.84 ± 3.18	0.26 ± 0.06	1.84 ± 0.10
11	5	12.22 ± 2.46	0.11 ± 0.02	179.29 ± 38.55	0.23 ± 0.05	1.83 ± 0.12
	10	15.06 ± 2.52	0.08 ± 0.01	189.44 ± 8.29	0.22 ± 0.03	1.86 ± 0.18
12	5	10.11 ± 1.97	0.13 ± 0.01	200.54 ± 8.64	0.28 ± 0.02	1.86 ± 0.38
	10	13.72 ± 2.66	0.06 ± 0.01	216.57 ± 3.06	0.25 ± 0.03	2.32 ± 0.56
13	5	12.92 ± 2.29	0.11± 0.01	198.64 ± 3.18	0.26 ± 0.06	1.85 ± 0.08
	10	15.25 ± 2.51	0.08 ± 0.02	214.83 ± 8.22	0.26 ± 0.02	2.14 ± 0.07
14	5	12.60 ± 3.25	0.08 ± 0.01	186.16 ± 3.18	0.25 ± 0.06	1.85 ± 0.07
	10	15.22 ± 2.58	0.06 ± 0.04	204.88 ± 4.12	0.26 ± 0.02	2.03 ± 0.11

*1: G6 + T0.02; 2: G6 + PG0.5; 3: G6 + A0.02; 4: G6 + T0.02PG0.5; 5: G6 + TA0.02; 6; G6 + PG0.5A0.02; 7: G6 + TA0.02PG0.5; 8: G3P3 + T0.02; 9: G3P3 + PG0.5; 10: G3P3 + A0.02; 11: G3P3 + T0.02PG0.5; 12: G3P3 + TA0.02; 13: G3P3 + PG0.5A0.02; 14: G3P3 + TA0.02PG0.5

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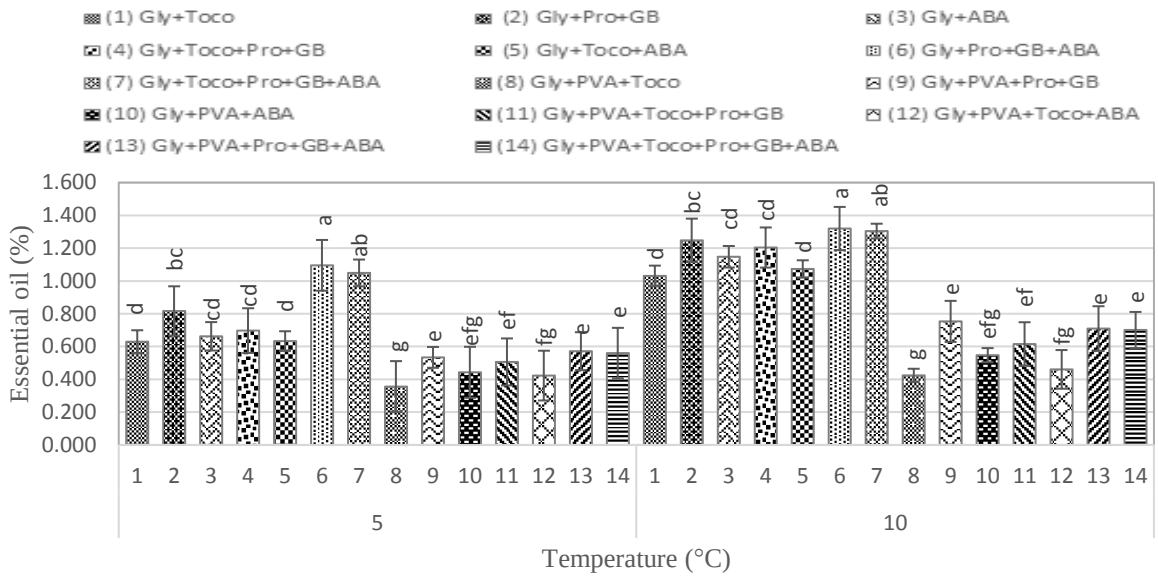

Fig. 1. Effect of anti-chilling formulations and adjuvants on essential oil content (%) of lemon verbena under different temperatures. Data given as means ± standard error, n =3. Error bars indicate standard error (SE). Gly: Glycerol, PVA: Polyvinyl alcohol, Toco: *α*-tocopherol, Pro: Proline, GB: Glycine betaine, ABA: Abscisic acid

4. Discussion

4.1. Effect of LTs

The temperature of 10 °C increased biomass traits and photosynthetic pigments content, while the antioxidant pigments and relative water content showed the similar effect at 5 °C. Our findings on leaf water content are in line with the results of research on rice [27]. According to previous findings, LT has induced anthocyanin synthesis in ray parenchyma cells of *Fagus sylvatica* due to their positive influence on photoprotection [28]. LT can interrupt processes of photosynthesis, including thylakoid electron transport, Calvin cycle, and stomatal conductance [29]. Except for polyphenols and enzymes activities, osmoprotectants showed the greatest content at 10 °C. The obtained results are in agreement with findings on the accumulation of sugar in rice at 8 °C [30]. During cold acclimation, carbohydrates function as nutrients and osmoprotectants and help plants survive in decreased temperatures [30]. Furthermore, plant growth regulators, e.g. PRO and spermidine, play a crucial role in the resistance to LT [31]. These findings are in line with previous studies exhibiting higher anti-oxidative enzyme activity in resistant plants compared to the LT sensitive ones [32, 33].

4.2. Effect of anti-chilling formulations

Morphological traits and relative water content augmented by application of G6 in comparison with G3P3. This result is in compliance with the results of experiment on cotton [34]. They showed that accumulation of GLY in osmotically stressed plant cells helps for maintaining plant water content. Treatment of G6 improved the content of photosynthetic pigments. GLY prevents the freezing of plant tissues by protective coating on the plant surfaces at LTs [35]. Contrary to SOD, polyphenols and MSI with the best result by G3P3, other enzymes and osmoprotectants showed the highest activity and content by G6. The foliar application of GLY can induce defense pathways by increasing the endogenous level of glyceraldehyde 3-phosphate [6].

In accordance with researches, the polarity and existence of OH groups in the chemical

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structures of polyol solutes such as GLY improve stress resistance in plants by osmotic adjustment, ROS scavenging, stabilization of macromolecular structures, and proteins protection against denaturation [36].

4.3. Effect of adjuvants (Auxiliary bioactive compounds)

The treatment of PG0.5A0.02 raised the morphological parameters and relative water content.

These results are in line with the study results on crop plants [37]. Foliar-applied GB improves crop production in stress conditions by translocation from the leaves to other plant organs [38].

The plants treated by TA0.02PG0.5 and TA0.02 showed the highest SPAD value and antioxidant pigment content, respectively. The obtained findings are in agreement with research results on the flax plant [39]. The application of adjuvants in the foliar-applied treatments on peach trees significantly improved leaf re-greening as compared to solutions alone [40]. The content of osmolytes was positively affected by PG0.5A0.02. Polyphenol content and MSI gained the best results by A0.02. These results are according to research results on mustard cultivars [41]. GB in mentioned treatments has an important role in the protection of several functional and tertiary structural proteins and vital enzymes (e.g. Rubisco), as well as membranes stabilization during freezing [38, 42]. PRO, essential for primary metabolism, acts as a signaling molecule to trigger specific gene expression essential for plant recovery against various types of abiotic stresses [43] TA0.02 and A0.02 resulting in the best activity of enzymes. TOCs are lipid-soluble antioxidants and are potential scavengers of ROS [44].

4.4. Effect of adjuvants in anti-chilling formulations under LTs

The rate of morphological parameters was increased at 10 °C, while the highest biochemical traits were observed at 5 °C. The greatest amount of morphophysiological traits was observed in treatments 6 and 5. This result is in accordance with the results of soybean plants under stress [45]. GLY as an osmolyte and inhibitor of cells freezing at LTs limits the loss of cellular water. These properties are related to strong hydrogen (H-) bonding between GLY and water [46]. According to research findings, exogenous application of ABA results in stomatal closure and reduces water loss via transpiration [47]. The antioxidant and photosynthetic pigments showed the highest content by treatment 5. These mentioned findings are similar to the results of research on tomato plants [48]. They showed increased photosynthesis of tomato plants by foliar application of GB. The protein content increased by foliar application of treatment 6. These results are in agreement with the reports on foliar application of GLY and defense reactions of *Theobroma cacao* [6]. GLY acts as a stabilizer of proteins under stress conditions. According to reports, protein stabilizers like glycerol cause a decrease in cold denaturation temperature and an increase in thermal denaturation temperature of proteins [49]. Findings of study on *Vicia faba* cultivars showed that the application of αTOC alleviated the injury of faba bean under stress conditions through the production of PRO and carotenoids and increasing enzyme activities, as well as protection of chloroplast membranes from photo-oxidation [50]. The rate of enzyme activities was increased at 5 °C. The best activity of enzymes was attained by treatment 3. Foliar application of ABA significantly increased the activities of SOD, CAT, and APX in maize seedlings under stress conditions. It has been shown that ABA application (before, during, or even shortly after an LT treatment) on LT

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sensitive plants improves the resistance to LT injury [51]. The content of osmolytes elevated at 10 °C. The highest content of TSS and PRO was observed in treatments 6, while the best result for polyphenol content and MSI was obtained by treatment 3. This result is in accordance with the results of experiment on *Vicia faba* plants under stress conditions [52]. They showed that exogenous application of PRO enhanced the content of total soluble solids. PRO may have a role in decreasing both the Na^+ content and Cl^- concentrations as well as increasing K^+ concentrations under stress conditions. Carbohydrates increased by foliar application of GLY and adjuvants act as osmoregulator, cryoprotectant, and signal molecules and also have an essential role as scavengers of reactive oxygen species to keep plants from LT injury [53]. This finding is similar to the results on yeast and *in vitro* conditions [54]. PRO is effective in protein and membrane stabilization, decreasing the T_m of DNA, and scavenging of reactive oxygen species. Exogenous GB has a benefit in inducing LTs tolerance in plants by increasing PRO accumulation [55]. G6 with PRO, GB and ABA mentioned as treatment 6 showed the best result on EO content. ABA has a protective and essential role in the stabilization of membranes and protection against oxidative stress. PRO acts as a signaling molecule to modulate mitochondrial functions, cell proliferation, or cell death [56].

Low surface tension causes an intimate contact between the leaf surface and the solution, as well as helps the spontaneous infiltration of stomatal cavities [57] and other possible hydrophilic domains of the cuticular layer. The adjuvants caused significant positive effects on the performance of treatments directly and indirectly. In addition to facilitating the leaf penetration process, they maintain treatments in a liquid form for a longer period due to their hygroscopicity (GLY and GB). Furthermore, they increase the tolerance of plants to stress conditions by synergistic interaction with other components of the formulation [57].

5. Conclusion

According to the interaction effects results, treatment glycerol + proline + glycine-betaine + ABA had the most positive effect on biomass, essential oil content, osmoprotectants of protein, proline, and TSS, while the best results of antioxidant pigments were obtained by treatment glycerol + α-tocopherol + ABA at 5 °C. Enzymes activities, polyphenol content, and membrane stability index showed the best results by application of treatment glycerol + ABA at LT. In conclusion, the treatments glycerol + ABA, and glycerol + proline + glycine-betaine + ABA is recommended as the best treatment in the reduction of the damaging effects of cold stress from the viewpoint of quality and quantity. These results showed the positive effect of adjuvants as well as anti-chilling formulations. Adjuvants increased the resistance of the plants against LTs both directly and indirectly. The direct and protective functions of proline, glycine-betaine, abscisic acid, and α-tocopherol are revealed in this study. Furthermore, they synergistically interact with other components of formulation as an indirect effect to combat the negative effects of LT. They also help in the leaf penetration process by lowering surface tension that enables direct contact between the leaf surface and the solution, as well as facilitating the spontaneous infiltration of stomatal cavities or maintaining treatments in a liquid form for a longer period due to their hygroscopicity.

Author contributions

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The first author, H.R. carried out the experiment and collected available literature and prepared the first draft of the manuscript with support from the fourth author; the second author, A.M. designed the model and the computational framework and he was also responsible for the correspondence; the third and fourth authors, H.N.Badi and F.Kh-S. analyzed the statistical data and verified the accuracy of the tests; the fifth and sixth authors, E.J., and N.R. edited the manuscript as biostimulant consultant.

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Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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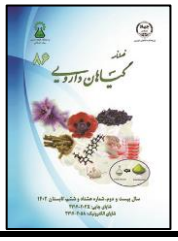

مقاله تحقیقاتی

کاهش آسیب دماهای پایین بر گیاه به لیمو (*Lippia citriodora* H.B.K.) با کاربرد خارجی اجونت‌ها و فرمولاسیون‌های ضدسرما

هانیه رفیعی[۱]، علی مهرآفرین[۲،۳*]، حسنعلی نقدی‌بادی[۴]، فرحناز خلیقی سیگارودی[۲]، اسماعیل جباری[۵]، نالینی راماوات[۶]

[۱] گروه علوم باغی و زراعی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران

[۲] مرکز تحقیقات گیاهان دارویی، پژوهشکده گیاهان دارویی جهاد دانشگاهی، کرج، ایران

[۳] مرکز تحقیقات گیاهان دارویی، دانشگاه شاهد، تهران، ایران

[۴] گروه زراعت و اصلاح نباتات، دانشکده کشاورزی، دانشگاه شاهد، آزاد راه تهران- قم، تهران، ایران؛ مرکز تحقیقات گیاهان دارویی، دانشگاه شاهد، تهران، ایران

[۵] آزمایشگاه‌های مهندسی بافت و مواد تقلید زیستی، گروه مهندسی شیمی، دانشگاه کارولینای جنوبی، کلمبیا، کارولینای جنوبی ۲۹۲۰۸، ایالات متحده امریکا

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اطلاعات مقاله

گل‌واژگان:
به لیمو
گلیسرول
پرولین
گلایسین بتائین
آلفا-توکوفرول
آبسیزیک اسید
سیتریک اسید

چکیده

مقدمه: به لیمو (*Lippia citriodora* H.B.K) از خانواده Verbenaceae یک گیاه حساس به سرما به عنوان تنش غیرزیستی است. **هدف**: هدف این تحقیق ارزیابی اثرات اجونت‌ها و فرمولاسیون‌های ضدسرما روی برگ‌های گیاه به لیمو (*Lippia citriodora* H.B.K) تحت تنش سرما بود. **روش بررسی**: تجزیه مرکب بر اساس طرح بلوک‌های کامل تصادفی (RCBD) با ۲۸ تیمار و ۳ تکرار انجام شد. سه فاکتور شامل ۲ فرمولاسیون ضد سرما (گلیسرول و گلیسرول + پلی وینیل الکل)، ۷ فرمولاسیون اجونت (آلفا- توکوفرول، آمینواسید پرولین + گلایسین- بتائین و اسید آبسیزیک) و ۲ سطح سرما (۵ و ۱۰ درجه سلسیوس) بودند. **نتایج**: تیمار گلیسرول + پرولین + گلایسین بتائین + اسید آبسیزیک آثار مخرب سرما را در بیوماس، محتوای اسانس و محافظت‌کننده‌های اسمزی کاهش داد در حالی که بالاترین میزان حفاظت از رنگدانه‌های آنتی‌اکسیدانی در تیمار گلیسرول + آلفاتوکوفرول + اسید آبسیزیک به‌دست آمد. فعالیت آنزیم‌ها و محتوای پلی‌فنل‌ها بهترین نتایج را با تیمار گلیسرول + اسید آبسیزیک نشان داد. **نتیجه‌گیری**: بهترین فرمولاسیون از دیدگاه بازده اقتصادی و همچنین حفظ کیفیت در برابر سرما به ترتیب گلیسرول + پرولین + گلایسین- بتائین + اسید آبسیزیک و گلیسرول + اسید آبسیزیک بود. کارایی فرمولاسیون‌های نامبرده به دلیل کارکرد محافظتی مستقیم و اثر غیرمستقیم اجونت‌ها در هم افزایی با دیگر ترکیبات فرمولاسیون بود.

مخفف‌ها: LTs، دمای پایین؛ RCBD، طرح بلوک‌های کامل تصادفی؛ SOD، سوپر اکسید دسموتاز؛ CAT، کاتالاز؛ APX، آسکوربات پراکسیداز؛ GLY، گلیسرول؛ PVA، پلی وینیل الکل؛ PRO، پرولین؛ GB، گلایسین- بتائین؛ EO، اسانس؛ αTOC، آلفا- توکوفرول؛ ABA، اسید آبسیزیک

* نویسنده مسؤول: Mehrafarin@imp.ac.ir

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