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Effect of bioregulators on the transpiration rate and photosynthetic index of sweet orange (*Citrus sinensis* (L.) Osbeck)

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Abstract
Sweet orange fruit is one of the best commercial fruits of India. 'Sathgudi' is a cultivar cultivated on large area in the Andhra Pradesh due to its reasonably higher yield, quality, taste and flavor. However, its average yield is decreasing year by year due to change in climate. Fruit drop is one of the main reasons for low citrus fruit yield, which is thought to be mainly due to hormonal imbalance in the plants. This imbalance may occur as a result of nutrient deficiency in orchard soils, water shortage, and insect pest attack on the citrus trees. Therefore, experiments were conducted to assess the influence of growth regulators (2,4-D and GA_3 and nutrients potassium and urea] to improve yield and quality of sweet orange fruit and control the fruit drop at two selected sites in the citrus growing tract of Andhra Pradesh. Foliar applications of 2,4-D, GA_3, potassium and urea significantly improved and have the beneficial effects on physiological parameters and photosynthetic index like relative water content of leaf, stomatal density, rate of transpiration, stomatal conductance, rate of photosynthesis, water use efficiency and chlorophyll content.

Keywords: Bioregulators, transpiration, photosynthetic, sweet orange

Introduction
Sweet orange (*Citrus sinensis* (L.) Osbeck), a second most important group of citrus is produced all over the world majorly in China, Brazil, India, Egypt, the European Union and Morocco. Sathgudi, is the choicest variety of sweet orange in Andhra Pradesh. Malnutrition of citrus orchards is a common problem and the scenario in Asian countries is of multiple natures. The poor fertilizer and bioregulators use efficiency is identified as one of the major causes of low orchard productivity. Fruit drop in citrus from marble stage of fruit development to harvest (Mohan *et al.* 1986) [18] is a serious problem worldwide and the size of the fruit is also an important, not only because it is a component of productive yield, but also determines the acceptance by the consumer. The application of plant growth regulators can re-enforce hormone balance in the peel, reducing or retarding this precocious fall and the losses at harvest (Primo *et al.* 1966) [21]. Citrus fruits use large amount of potassium (Liu *et al.* 2000) [15]. It improves fruit quality through enhancing fruit colour, size and juice flavor (Tiwari 2005) [28]. Citrus production has also been maintained by applying 3–6 foliar applications of urea per year implying that 16 to 33% of the annual requirement of citrus could be supplied with a single foliar application. There is a need to boost up yield through proper nutrition and maintaining internal hormonal balance. Suitable combination of nutrients and growth regulators may control excessive fruit drop for the improvement of fruit yield and quality. Although many efforts have been made to study the physiological and biochemical aspects of citrus, there is still an enormous unexplored potential in the study of regulation of metabolites associated with citrus physiology.

Materials and Methods
This investigations, was conducted at the experimental field of Citrus Research Station, Tirupati, Department of Fruit Science, Dr. Y.S.R. Horticultural University, in Chitoor District (Location-1) and also at farmer's field of Railway Kodur in Kadapa District, (Location-2) of Andhra Pradesh during the year 2018 to 2019. The experiment was conducted in randomized block design with three replications and two trees for each replication. The experiment involved following ten treatments.

T_1 Monopotassium phosphate @ 1% + 2, 4-D @ 10 ppm (spray one month after fruit set)
T_2 Urea @ 1% + 2,4-D @ 10 ppm (spray one month after fruit set)
T_3 Monopotassium phosphate @ 1% + GA_3 @ 10 ppm (spray one month after fruit set)
T_4 Urea @ 1% + GA_3 @ 10 ppm (spray one month after fruit set)
T_5 Monopotassium phosphate @ 1.5% + 2,4-D @ 10 ppm (spray one month before harvest)
T_6 Urea @ 1.5% + 2, 4-D @ 10 ppm (spray one month before harvest)
T_7 Monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm (spray one month before harvest)
T_8 Urea @ 1.5% + GA_3 @ 10 ppm (spray one month before harvest)
T_9 Waste Decomposer @ 200 L/acre (spray before flowering)
T_{10} Farmers Practice: (Urea 1% + Multi K (Potassium Nitrate) 1% @ 10 g/L (spray one month after fruit set)

Relative water content of leaf (%)

It is a reliable trait, for screening drought tolerance (Rachmilevitch *et al*. 2006) [22]. It is defined as the percentage of water present at the time of sampling, relative to the amount of water in a saturated leaf.

Twenty, one square centimeter discs of leaves were collected from all over the canopy, were quickly weighed to record the fresh weight then they were made to float on water in the dark at room temperature (25 °C) for 24 hours then they are weighed to record the turgid weight. RWC was determined after drying the leaf discs at 60 ºC for 2 days for dry weight and was calculated as follows.

RWC (%) = Fresh weight - Dry weight / Turgid weight- Dry weight x 100

Stomatal Density (No./mm^2)

The leaf lamina was treated with acetic-formalin-alcohol and tangential sections of the leaf blades were studied under DMi8 Leica inverted microscope from Wetzlar, Germany Leica Microsystems. Dilute solution of potassium hydroxide is used to remove hesperidin and 90 per cent alcohol to remove the chlorophyll, then stained with alcoholic safronin and finally cleared in dilute potassium hydroxide. The number of stomata in area of 1 mm^2 was counted and the average of ten or more areas was recorded. Areas in the vicinity of oil glands, epidermal hairs and large veins were avoided (Reed and Hirano, 1931) [23].

Rate of transpiration (mol of H_2O/cm^2/s), stomatal conductance (mmol/m^2/s) and rate of photosynthesis (µg CO_2 /m^2/s)

All these three parameters were measured for three newly formed mature leaves which are fully exposed to Sun from all the four sides of the tree branches. This was done in the morning from 8:00 AM to 10:00 AM using artificial sunlight (1000 µEim m^{-2} sec^{-1}) with portable photosynthetic meter with light control (Licor, Model-LI 6400).

Water use efficiency (kg/ha/cm)

The ratio of photosynthesis and rate of transpiration gives the water use efficiency.

Change in leaf dry weight (mg/cm^2/h^2)

Determination of photosynthetic index on dry weight basis for comparative study of different treatments was done in accordance with this procedure.

To determine the change in leaf dry weight, ten leaves at random on each experimental tree were tagged and 20 leaf discs of 1 cm^2 diameter with a cork borer from one side of these leaves in the morning were taken and dried in an oven for 24 hours at 70 °C. Discs from other side of the midrib of the same leaves were removed in the evening 10 hours after first removal and were dried as those of morning discs. After ensuring total driage of both sets of leaf discs, their dry weight was recorded. Any increase in weight was recorded. Any increase in weight in evening discs over morning discs was attributed to accumulation of photosythates synthesized during 10 hours and expressed as mg cm^{-2} h^{-2}

SPAD chlorophyll meter reading (SCMR) (µmol of chlorophyll per m^2)

The SCMR was measured on mature active leaves. SPAD meter of Minolta, NJ, USA (SPAD 502).

Results and Discussion

Physiological parameters

Relative water content of leaf (%)

Among the ten treatments, evaluated at both the locations (table – 1a), mean relative water content of leaf revealed that, plants that were sprayed with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm, one month before harvest has recorded maximum (53.65%) while, the minimum (36.17%) was recorded with monopotassium phosphate @ 1% + GA_3 @ 10 ppm, sprayed one month after fruit set.

It is an important indicator of water status in plants, which reflects the balance between water supply to the leaf tissue and transpiration rate Lugojan and Ciulca, (2011) [16]. The reductions in uptake of water and transpiration are usually associated with a reduction in the water content of the shoots and stomatal aperture, suggesting that water stress has developed in the leaves Gerakis *et al*, (1975) [6]. Potassium, which was sprayed one month before harvest affects both uptake of water through plant roots and its loss through the stomata. It is also known to improve drought resistance by maintaining higher ratio of unsaturated/saturated fatty acid which plays a major role in imparting drought tolerance, regulation of internal water balance and turgidity. These results are in concord with Diego and Manuel (2006) [4] as he reported that plants receiving adequate potassium nutrition displayed greater relative water content of leaf in olive.

Stomatal density (Number of stomata/mm^2)

Mean stomatal density among the treatments showed significant difference (table – 1a) in two locations and it was observed as lowest (133.99 stomata per mm^2) in plants which were sprayed with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm, one month before harvest and the highest (246.86 stomata per mm^2) in plants sprayed with urea @ 1% + 2,4-D @ 10 ppm, one month after fruit set.

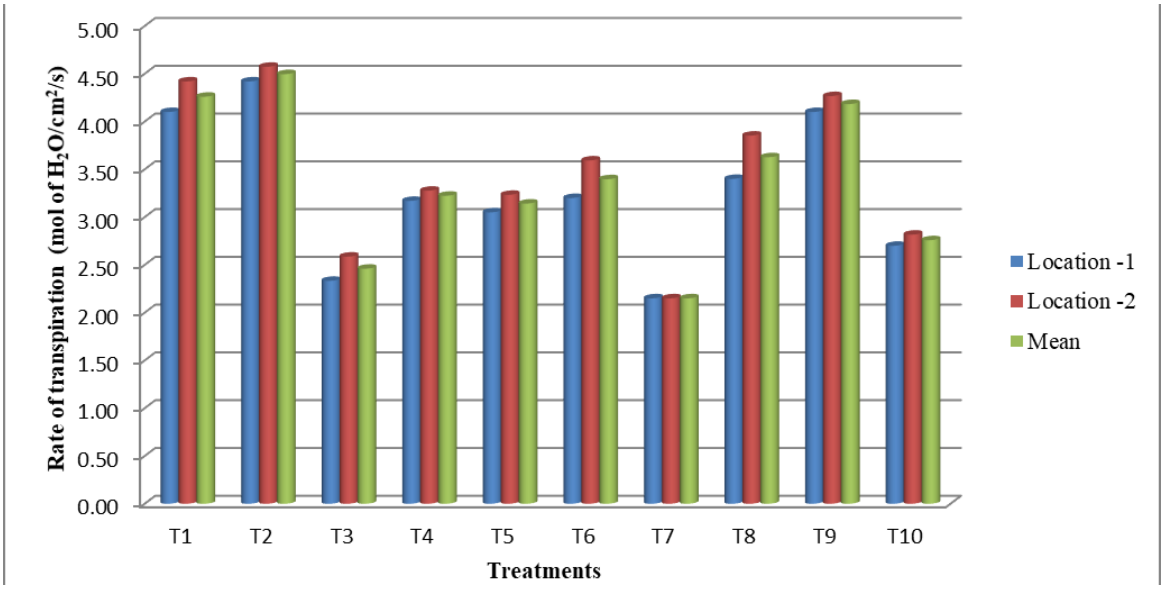

Fig 1: Effect of bioregulators on rate of transpiration (mol of $H_2O/cm^2/s$) in sweet orange cv. Sathgudi

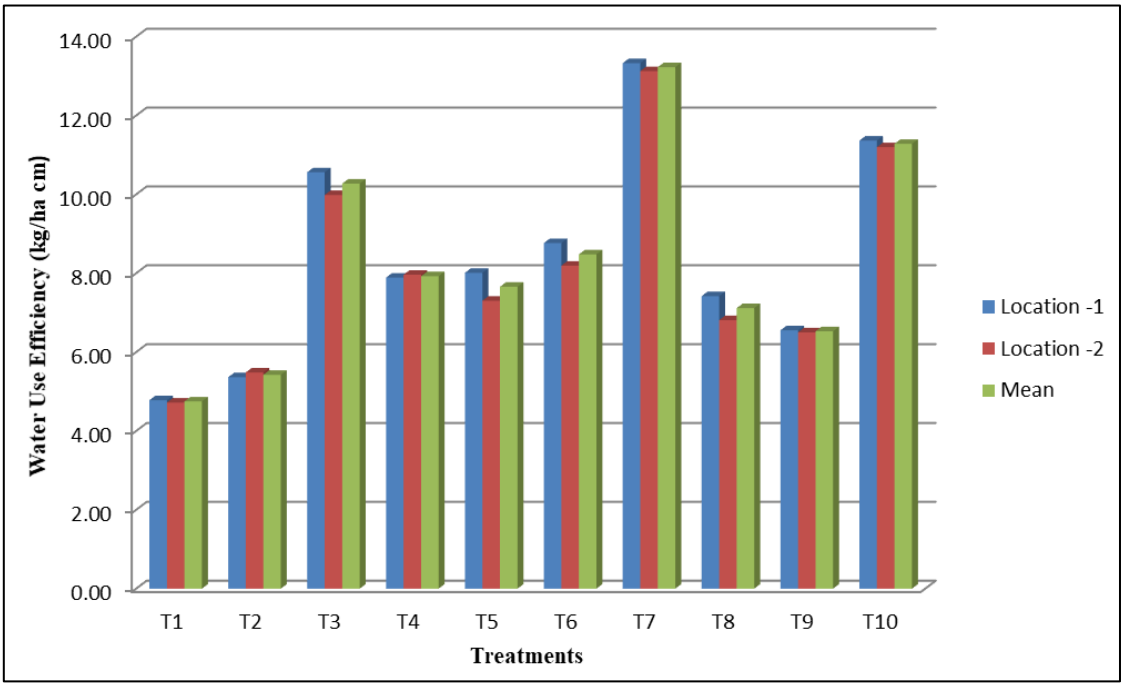

Fig 2: Effect of bioregulators on water use efficiency (kg/ha cm) in sweet orange cv. Sathgudi

Table 1a: Effect of bioregulators on physiological parameters of sweet orange (*Citrus sinensis* L. Osbeck)

	Relative water content of leaf (%)			Stomatal density (per mm^2)			Rate of transpiration (mol of $H_2O/cm^2/s$)		
	Experimental locations			Experimental locations			Experimental locations		
Treatments	**Location - 1**	**Location - 2**	**Mean**	**Location - 1**	**Location - 2**	**Mean**	**Location - 1**	**Location - 2**	**Mean**
T1	36.71	42.12	39.42	228.66	232.82	230.74	4.10	4.42	4.26
T2	35.30	37.04	36.17	244.58	249.13	246.86	4.42	4.57	4.50
T3	49.18	50.35	49.76	179.27	162.06	170.67	2.33	2.58	2.46
T4	36.63	39.34	37.99	209.14	253.73	231.44	3.17	3.27	3.22
T5	51.26	52.01	51.63	145.71	182.02	163.87	3.05	3.23	3.14
T6	48.87	49.10	48.98	164.10	190.75	177.43	3.20	3.59	3.40
T7	52.81	54.50	53.65	136.16	131.81	133.99	2.15	2.15	2.15
T8	46.27	48.67	47.47	157.20	217.41	187.31	3.40	3.85	3.63
T9	44.04	45.59	44.82	158.10	149.59	153.84	4.10	4.26	4.18
T10	46.82	47.58	47.20	152.52	151.63	152.08	2.70	2.81	2.76
SE(m)+	2.19	1.75	1.83	5.54	11.80	6.71	0.14	0.15	0.11
CD	6.57	5.25	5.48	16.60	35.34	20.11	0.42	0.45	0.34
Min	35.30	37.04	36.17	136.16	131.8	133.99	2.15	2.15	2.15
Max	52.81	54.50	53.65	244.58	253.73	246.86	4.42	4.57	4.50

Table 1b: Effect of bioregulators on physiological parameters of sweet orange (*Citrus sinensis* L. Osbeck)

	Stomatal conductance (mmol/m^2/s)			Water use efficiency (kg/ha cm)		
	Experimental locations			Experimental locations		
Treatments	Location – 1	Location - 2	Mean	Location - 1	Location - 2	Mean
T1	0.004	0.005	0.005	4.78	4.72	4.75
T2	0.004	0.005	0.005	5.36	5.48	5.42
T3	0.002	0.003	0.003	10.56	9.98	10.27
T4	0.003	0.004	0.004	7.89	7.96	7.92
T5	0.004	0.004	0.004	8.01	7.30	7.65
T6	0.003	0.004	0.003	8.76	8.19	8.48
T7	0.010	0.012	0.011	13.32	13.12	13.22
T8	0.016	0.017	0.017	7.42	6.81	7.11
T9	0.019	0.020	0.020	6.55	6.50	6.52
T10	0.003	0.003	0.003	11.36	11.19	11.28
SE(m)+	0.000	0.000	0.000	0.44	0.54	0.39
CD	0.001	0.001	0.001	1.32	1.64	1.17
Min	0.002	0.003	0.003	4.78	4.72	4.75
Max	0.019	0.020	0.020	13.32	13.12	13.22

Table 2: Effect of bioregulators on photosynthetic index of sweet orange (*Citrus sinensis* L. Osbeck)

	Change in leaf dry weight (mg/cm^2/h^2)			Chlorophyll content (SCMR) (µmol of chlorophyll per m^2)			Rate of Photosythesis (µg CO_2 /m^2/s)		
	Experimental locations			Experimental locations			Experimental locations		
Treatments	Location - 1	Location - 2	Mean	Location - 1	Location - 2	Mean	Location - 1	Location - 2	Mean
T1	0.025	0.036	0.030	65.26	68.90	67.08	19.50	20.83	20.16
T2	0.045	0.043	0.044	67.86	66.70	67.28	23.60	24.93	24.26
T3	0.042	0.044	0.043	67.40	73.90	70.64	24.60	25.30	24.95
T4	0.054	0.052	0.053	66.60	64.63	65.61	24.90	25.83	25.36
T5	0.045	0.047	0.046	67.53	70.36	68.96	24.40	23.56	23.98
T6	0.050	0.050	0.050	68.96	68.13	68.55	27.40	29.40	28.40
T7	0.065	0.064	0.065	66.00	75.43	70.71	28.60	28.10	28.35
T8	0.014	0.015	0.014	67.86	69.80	68.83	25.20	26.20	25.70
T9	0.033	0.035	0.034	65.46	74.20	69.83	26.80	27.60	27.20
T10	0.019	0.025	0.022	66.10	63.50	64.80	30.60	31.30	30.95
SE(m)+	0.001	0.001	0.001	-	1.88	1.09	0.87	0.68	0.52
CD	0.002	0.003	0.002	NS	5.62	3.27	2.61	2.03	1.56
Min	0.014	0.015	0.014	65.26	63.50	64.80	19.50	20.83	20.16
Max	0.065	0.064	0.065	68.96	75.43	70.71	30.60	31.30	30.95

Stomata close in response to various environmental stresses and it involves the phytohormones Schroeder *et al.* (2001) [25]. Guard cells extrude H^+ ions during stomatal opening in the presence of light and take up K^+ and Cl^- and produce $malate^{2-}$ Shimazaki *et al.* (2007) [26]. This uptake and efflux pathways across the tonoplast are vital for stomatal responses. Anion release and Ca^{2+} uptake depolarize the plasma membrane which shifts the membrane potential more towards positive values. This depolarization provides the driving force for a decrease in K^+ and $malate^-$ which causes guard cells to release water and shrink, thus relaxing the outward bend and closing over the substomatal cavity (Kearns and Assamann, 1993 and Blatt *et al.* 1993) [10]. Brag in (1972) [2] reported that plants with high amounts of potassium were found to have the lowest stomatal frequency in the leaves.

The opening and closing responses of stomatal guard cells are also accomplished by the hormonal control of ion channel gating in response to various environmental stresses. Apart from ABA, phytohormones such as gibberellins also have a role in stomatal regulation of gaseous exchange during water stress. Recent studies of GA-responsive transcriptome have demonstrated the involvement of GA in biotic and abiotic stress tolerance. Goh *et al.* in (2009) [7] reported that GA_3 regulates the inhibition of light induced stomatal opening by ABA in a concentration-dependent manner in *Arabdiopsis thaliana*. ABA regulates a set of ion channels and H^+ pumps in guard cells that are associated with fast stomatal closure. This stress hormone has a large impact on gene activation or inactivation in several cell types (Leonhardt *et al.* 2004 and Levchenko *et al.* 2007) [12, 13]. The expression of the ABA - inducible gene, RD22 may also be associated with the response to GA_3. The expression of the RD22 gene increased in response to ABA treatment when plants were grown in the absence of GA_3 however, this expression was reduced over time in plants that were grown in the presence of GA_3. The expression of the GA inducible genes, GASA4 and AtEXP1, was reduced in plants that were treated with ABA. So they predicted that GA_3 inhibits ABA signaling during stomatal closing.

Rate of transpiration (mol of H_2O/cm^2/s)

Mean rate of transpiration was significant (table – 1a) among the treatments and the lowest (2.15 mol of H_2O/cm^2/s) was recorded in plants that were treated with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm, one month before harvest while the highest rate (4.50 mol of H_2O/cm^2/s) was recorded in plants that were treated with urea @ 1% + 2, 4-D @ 10 ppm, one month after fruit set.

Potassium is required for translocation of assimilates, involved in maintenance of water status of plants especially the turgor pressure of cells, osmoregulation and opening and closing of stomata. According to Levitt (1974) [14], proton-transport concept, the opening and closing of the stomata are the results of an active transport of potassium ions into the

guard cells and out of them. The adjacent epidermal cells act as ion storage cells for guard cells. Brag (1972) [2] reported that plants with high amounts of potassium were found to have the lowest transpiration rates. GA_3 also helps in regulation of genes responsible for stomatal closure. As the rate of transpiration depends upon stomatal density, the lowest stomatal density in the same treatment might be the reason for lowest transpiration. These results were also in agreement with Diego and Manuel (2006) [4] as he reported that plants receiving adequate potassium nutrition displayed lower transpiration in olive.

Stomatal conductance (mmol/m^2/s)

Stomatal conductance is the measure of the rate of passage of carbon dioxide (CO_2) entering, or water vapor exiting through the stomata of a leaf.

Means over two locations was significant (table – 1b) and was observed as the highest in the plants that were sprayed with waste decomposer @ 200 L/acre before flowering (0.020 mmol/m^2/s) and the lowest (0.003 mmol/m^2/s) was recorded in plants that were sprayed with monopotassium phosphate @ 1% + GA_3 @ 10 ppm one month after fruit set, urea @ 1.5% + 2, 4-D @ 10 ppm sprayed one month before harvest and also in farmer's practice of spraying urea @ 1% + multi K (Potassium Nitrate) 1% @ 10 g/L one month after fruit set.

Dynamic regulation of stomatal aperture requires large quantities of potassium to be rapidly exchanged between apoplast, cytosol and vacuoles of stomatal guard cells (Roelfsema and Hedrich 2008, Andres *et al.* 2014) [24, 1]. Transport of potassium ions through the plasma membrane of stomatal guard cells is mediated by voltage gated inward rectifying K^+ channels and is accompanied by influx of counter ions (NO^{3-}, Cl^-) and synthesis of malate in the cytosol. Increased concentrations of ions decrease the guard cell water potential and cause an influx of water. As a result, guard cell volume increases and the stomata open. Due to the osmotic role of potassium in guard cell regulation, it is not surprising that stomatal conductance is frequently reported to decrease under potassium deficient conditions (Jakli *et al.* 2017) [8]. Nevertheless and regardless of the importance of potassium in guard cell regulation, changes in stomatal conductance do not limit photosynthesis predominantly, even when total leaf potassium concentrations are below the critical level for photosynthesis (Zhao *et al.* 2001, Jin *et al.* 2011) [29, 9]. These results are in line with the work of Diego and Manuel (2016) in olive.

Water use efficiency (kg/ha/cm)

Water use efficiency refers to the ratio of water used in plant metabolism to water lost by the plant through transpiration.

Mean data for two locations as shown on table – 1b was significant and was maximum (13.22 kg/ha/cm) in plants treated with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm one month before harvest while, minimum (4.75 kg/ha/cm) in plants treated with monopotassium phosphate @ 1% + 2,4-D @ 10 ppm one month after fruit set.

Water use efficiency is the most important strategy evolved by plants to survive under water limited conditions. It is computed that an increase in WUE by even 0.1 unit can result in enhancement of total biomass production by about 0.32 tonnes per hectare. Variation in WUE is brought about by stomatal diffusion character and photosynthetic capacity. Plants have evolved to maximum WUE through reduction in transpiration that is liked with stomatal conductance. As number of stomata and rate of transpiration were less in plants sprayed with monopotassium phosphate 1.5% + GA_3 10 ppm one month before harvest, the water use efficiency was highest in those plants sprayed with the same treatment. These results are in harmony with Diego and Manuel, (2006) [4] where he reported that plants receiving adequate potassium nutrition displayed greater water use efficiency in olive. Similar results were also recorded by Lakshmi *et al.* (2019) [11] where the maximum WUE of 0.82 t/ha-cm was observed in irrigation schedule at 70% ER along with fertigation level 80% RDF (I1F3) in 'Sathgudi' sweet orange.

Photosynthetic Index

Change in leaf dry weight (mg/cm/h)

Mean change in leaf dry weight among the treatments in two locations as shown in table – 2 was observed to be significant and recorded to be highest (0.065 mg/cm/hr) in plants that were sprayed with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm, one month before harvest and lowest (0.014 mg/cm/hr) in plants that were sprayed with urea @ 1.5% + GA_3 @ 10 ppm one month before harvest.

Chlorophyll content (µmol of chlorophyll per m^2)

Mean data for chlorophyll over the two locations (table – 2) was highest (70.71 µmol of chlorophyll per m^2) in plants sprayed with monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm spray one month before harvest while lowest (64.80 µmol of chlorophyll per m^2) was recorded in plants sprayed with farmer's practice of spraying urea 1% + Multi K 1% @ 10 g/L one month after fruit set.

Li *et al.* (2018) reported that chlorophyll might be an indicative trait for characterizing how plants respond to climate change. Chlorophyll synthesis requires many elements like nitrogen, phosphorous and water. GA_3 also influences chlorophyll content of leaves and this was evident from the experiments done by El-Gioushy *et al.* (2018) [5], where they observed that total chlorophyll content of fresh leaves increased in response to the different increasing concentrations of GA_3 (0, 25 and 50 ppm) and they reported that highest chlorophyll content was obtained with highest GA_3 concentration *i.e.* 50 ppm in 'Washington Navel' orange trees budded on sour orange rootstock.

Rate of photosynthesis (µg CO_2 /m^2/s)

Photosynthesis is the most important source of energy for plant growth.

Mean values of two locations as shown in table – 2 was significant and maximum (30.95 µg CO_2/m^2/s) in farmer's practice of spraying urea 1% + Multi K (Potassium Nitrate) 1% @ 10 g/L one month after fruit set and minimum (20.16 µg CO_2/m^2/s) was found in plants sprayed with monopotassium phosphate 1% + 2,4-D 10 ppm one month after fruit set.

Rate of photosynthesis was influenced by number of external like light quality and intensity, CO_2 concentration, temperature, oxygen, water, wind and nutrient level. Besides these certain internal factors like chlorophyll content, stomatal behaviour, concentration of carboxylating enzymes and leaf water content influence the photosynthetic rate. All these internal factors are high for the treatment having high rate of photosynthesis. Merle *et al.* (2018) [17] reported that adequate K is required to maintain the photosynthetic activity of crops (eg. Cooper *et al.* 1966, Terry and Ulrich 1973, Peoples and Koch 1979) [3, 27, 20]. Below a certain threshold of leaf K,

assimilation rates drop drastically. The tissue potassium concentration is critical for photosynthetic functioning of crops.

Conclusion

Application of monopotassium phosphate @ 1.5% + GA_3 @ 10 ppm spray one month before harvest has given best results for decreasing the rate of transpiration and thereby increasing the photosynthetic characters which can improve the physiology of plants and increase the productivity of trees by preventing fruit drop caused due to changing climate.

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