Saudi Journal of Biological Sciences 27 (2020) 318–323

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Analysis of the cuticular wax composition and ecophysiological studies in an arid plant - *Ziziphus nummularia* (Burm.f.) Wight & Arn.

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ARTICLE INFO

Article history:
Received 14 April 2019
Revised 22 September 2019
Accepted 29 September 2019
Available online 16 October 2019

Keywords:
Cuticle
Ecophysiology
Photosynthesis
Quantum yield
Transpiration

ABSTRACT

Plants in arid regions are exposed to various abiotic stresses and the presence of the waxy cuticular layer acts as a defensive barrier, which consists mainly of long chain fatty acids, hydrocarbons and other derived compounds. Studies on the chemical composition and properties of cuticles of arid plants are scanty. The present study deals with the analysis of cuticular wax composition and effect of temperature on some ecophysiological parameters of an important arid plant *Ziziphus nummularia*. A total of 59 different wax compounds were detected from the leaf cuticle by capillary GC–MS. 4-Hydroxycyclohexanone, Heptacosane and 2,7-Dimethyloctane-3,5-dione were the dominant wax compounds in *Z. nummularia*. The variation of photosynthetic rate varied from 0.70 to 7.70 μmol CO2 m-2s-1 against the studied temperature range of 15–55 °C. The transpiration rate varies from 1.80 to 8.40 mmol H2O m-2s-1 within the temperature range of 15–55 °C. The quantum yield of photosystem II (Fv/Fm) also exhibited much variation due to the variation of temperature. The results clearly shows that *Z. nummularia* is highly adapted to restrict water loss and can tolerate high temperatures and can be considered as an appropriate species for vegetating the arid areas.

1. Introduction

The arid life is prone to prolonged water deficit, which poses problems to all organisms, especially plants, which have developed a multitude of properties and strategies letting them to survive with water scarce conditions and concomitant water losses. The demands on the water relations of desert plants are intensified by high temperature and low air humidity in many Saudi Arabian desert areas, which tremendously increase the driving force for water loss to the atmosphere. The characteristic features of desert plants to thrive in their natural living conditions should be considered as an important area of research in order to identify the primary traits that provide resistance to arid habitat. The surfaces of the aerial parts of plants are protected by a thin layer of cuticle which helps to check the uncontrolled non-stomatal loss of water into the surrounding atmosphere. The cuticle of plants is made up of a cutin matrix which is seen embedded with cuticular waxes (Pollard et al., 2008; Yeats and Rose, 2013). Chemically, cutin is a polymer of ester-linked ω-hydroxylated fatty acids, whereas cuticular wax is a complex blend of long chain fatty acids, terpenoids and flavonoids (Fei et al., 2018; Jeffree, 2006). Jetter et al. (2006) has reported that cuticular waxes contains *n*-alkanes, primary alkanols, alkanoic acids, alkanals, alkyl esters etc in addition to pentacyclic triterpenoids mainly of the oleanane, lupane and ursane types. It is the presence of these wax compounds in the plant cuticles that defines the transpiration barrier properties (Schuster et al., 2017).

Ziziphus nummularia belonging to the family Rhamnaceae is one of the most commonly occurring drought hard thorny shrub species in the arid and semi-arid regions. It is an intricately branched shrub with small oval leaves and paired spines which attains a height of 1–3 m and has light colored bark. As a plant adapted to arid environment, *Ziziphus nummularia* can tolerate various abiotic stresses, such as high temperature, drought and salinity. The chemical profiling of the cuticle of *Ziziphus nummularia* is not reported before.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.09.030
1319-562X/

An extremely small number of reports concerning the chemical properties of cuticles of arid plants are only available till now. Many of these studies are purely descriptive and are devoted exclusively to the anatomical features of the epidermis. Apart from studying the cuticular composition, the present study also focusses on the effect of temperature on the photosynthetic rate, quantum yield and rate of transpiration in *Z. nummularia*.

2. Materials and methods

2.1. Leaf materials

Leaves of *Ziziphus nummularia* were collected from an original growing area in Rhaudat Al-Khuraim, located about 100 km north-east of Riyadh in the Kingdom of Saudi Arabia (Fig. 1). *Z. nummularia* locally known as 'Sidr' is a highly branched shrub with ovate to orbiculate finely haired leaves. Fully developed and intact leaves were harvested and transferred to the laboratory.

2.2. Scanning electron microscopy of leaf surfaces

The ultrastructure of the abaxial and adaxial leaf surfaces of *Z. nummularia* was characterized using scanning electron microscopy. The leaves were air dried, made into small pieces, fixed to aluminum holders, sputter-coated with gold palladium alloy and imaged with SEM (Dragota and Riederer, 2007).

2.3. Analysis of cuticular wax composition by GC–MS

The extraction of plant cuticular waxes has been done according to a standard protocol (Zabka et al., 2008). For determining the cuticular wax composition by GC–MS the protocol described by Dragota and Riederer (2007) was followed.

2.4. Gas exchange parameters and chlorophyll fluorescence

The steady state CO_2/H_2O gas exchange parameters were performed using a portable photosynthesis system and chlorophyll fluorescence was determined with the help of a fluorometer following the methodology proposed by Baker and Rosenqvist (2004). The efficiency of photosystem II (F_v/F_m) was calculated using the ratio of the minimum fluorescence level of dark-adapted leaves and the maximum fluorescence (Schreiber et al., 1995).

Fig. 1. *Ziziphus nummularia* plant with leaves and fruits (Photo courtesy: Dr. Jacob Thomas Pandalayil).

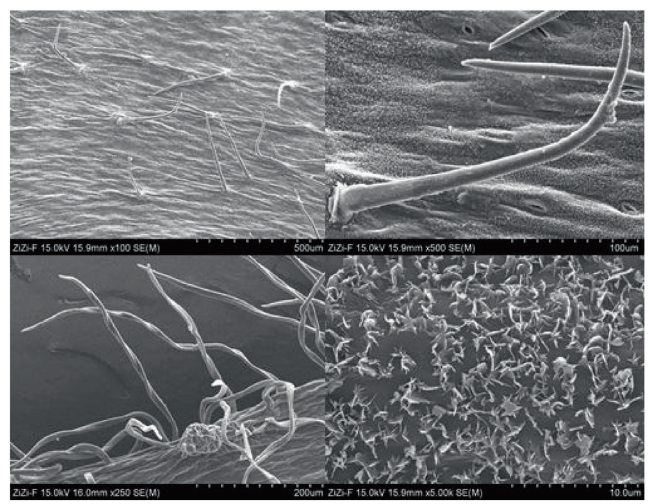
3. Results

3.1. Leaf surface properties of Ziziphus nummularia

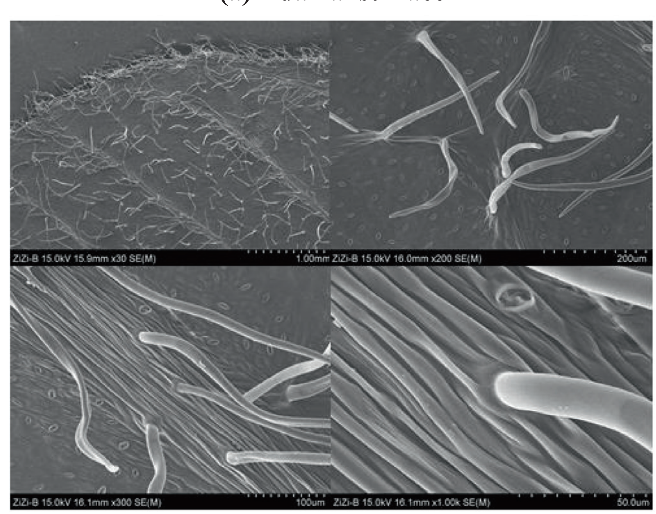
The leaves of *Ziziphus nummularia* contain stomata on both the adaxial and the abaxial leaf surfaces (Fig. 2). Stomata are anisocytic and sunken. The mesophyll layer consists of 3 to 4 layers of long palisade cells on the adaxial surface and 2 to 3 layers of small cells on the abaxial surface. Both leaf surfaces are pubescent in nature having simple short unicellular, thin walled trichomes.

3.2. Composition of wax compounds in Ziziphus nummularia

The composition of wax compounds in the leaves of *Ziziphus nummularia* was analyzed and a total of 62 different wax compounds were detected. The chemical formula, molecular weight, retention time and the individual amount (%) of different wax compounds of *Ziziphus nummularia* are presented in the Table 1. The wax compounds 4-Hydroxy-cyclohexanone, Heptacosane and 2,7-Dimethyloctane-3,5-dione were dominated over other wax compounds. The amount of these three compounds i.e., 4-Hydroxy-cyclohexanone, Heptacosane and 2, 7-Dimethyloctane-3, 5-dione were 16.152%, 14.381% and 9.342%, respectively.



(a) Adaxial surface



(b) Abaxial surface

Fig. 2. The morphology of the untreated leaf surfaces of *Ziziphus nummularia*.

Table 1
Summary of the analysis of wax compounds of *Ziziphus nummularia.*

Peak	Compound name	Formula	MW	Retention time	Area (%)
1	4-Hydroxy-cyclohexanone	C6H10O2	114.145	8.415	16.152
2	Ethyl tigalate	C7H12O2	128.170	8.776	1.251
3	2,5-Dipropyltetrahydrofuran	C6H20O	156.269	9.056	2.229
4	1,1-Dimethoxycyclohexane	C8H16O2	144.211	9.543	0.274
5	2,6-Dimethyl-3,5-heoptanedione	C9H16O2	156.222	13.666	0.688
6	6-Dodecanone	C12H24O	184.318	14.758	1.171
7	1-Cyclohexyl-2,2-dimethyl-1-propanol	C11H22O	170.290	15.101	0.405
8	1,1-Diethoxy-2-hexene	C10H20O2	172.264	16.112	0.674
9	2,7-Dimethyloctane-3,5-dione	C10H18O2	170.248	16.334	9.342
10	5-propylnonane	C12H26	170.335	16.507	1.526
11	2-Methyl-2-propenoic acid 1,2-ethanediyl ester	C10H14O4	198.216	17.028	0.719
12	1,2-Cyclobutanedicaboxylic acid 3-methyl- dimethyl ester	C9H14O4	186.000	17.159	0.668
13	1-Hexene-3,5-dione	C6H8O2	112.127	17.510	0.370
14	Trans-1,10-Dimethyl-*trans*-9-decalinol	C10H18O2	182.307	17.720	2.839
15	2,2,6-trimethylheptane-3,5-dione	C12H22O	170.248	17.891	0.774
16	3-Methyldodecane	C12H28	184.361	20.732	0.386
17	2-Methyl-5-propylnonane	C13H28	184.367	21.319	0.618
18	3-Methyl-5-propylnonane	C13H28	184.367	21.969	0.428
19	5-ethyl-5-methyldecane	C13H28	184.361	22.217	0.620
20	4.6-Dimethyl dodecane	C14H30	198.394	22.802	6.941
21	4-Methoxycarbonyl 4-pentenoic acid isopropy ester	C10H16O4	200.104	23.023	0.800
22	2,6,11-Trimethyldedecane	C15H32	212.414	23.858	0.272
23	Undecyl acetate	C13H26O2	214.344	24.005	3.163
24	Hexadecane	C16H34	226.000	27.578	0.561
25	2,2,4,4,6,8,8-Heptamethylnaonane	C16H34	226.448	27.667	0.648
26	2.6.10-Trimethyl tetradecane	C17H26	240.467	27.983	0.835
27	7,9-Dimethylhexadecane	C18H38	254.297	28.046	1.486
28	4-Methyl heptadecane	C18H38	254.490	28.407	0.615
29	Octadecane	C18H38	254.493	29.075	0.859
30	2,6,10,14-Tetramethylhexadecane	C20H42	282.548	29.223	2.569
31	5-(Tetrahydro-2–furanylmethyl)-2-heptanol	C12H24O	200.318	29.335	0.786
32	2,4-Di-*tert*-butylphenol	C14H22O	206.167	29.463	0.251
33	2,6,11,15-Tetramethylhexadecane	C20H42	282.556	29.659	0.252
34	10-Methyl-8-tetradecen-1-ol acetate	C17H32O2	268.441	29.716	
35	2.3.3-Trimethyl-2-(4-methylpentanoyl)-cyclopentanore	C14H24O2	224.000	29.896	
36	2-Methyleicosane	C21H44	296.344	30.176	
37	*trans*-2-hexadecenoic acid	C16H30O2	254.400	30.852	0.368
38	Heneicosane	C21H44	296.583	31.327	
39	Docosane	C22H46	310.601	32.273	3.178
40	1,5-Dicyclopentyl-3-(2-cyclopentylethyl)pentane	C22H40	380.745	32.931	0.696
41	Tetracosane	C24H50	338.650	33.225	0.328
42	Pentacosane	C25H52	352.680	33.549	1.669
43	Hexacosane	C26H54	366.710	34.015	0.288
44	7-Hexylicosane	C26H54	366.707	34.315	0.220
45	10-Methyl-8-tetradecen-1-ol acetate	C17H32O2	268.441	35.530	1.178
46	Hexadecanoic acid methyl ester	C17H34O2	270.450	35.671	
47	*trans*-3-pentyl oxiraeundecanoic acid methyl ester	C19H36O2	312.480	35.719	0.274
48	Phytol acetate	C22H42O	338.568	35.739	0.238
49	Ingol 12-acetate	C22H32O7	408.491	35.889	
50	Heptacosane	C27H56	380.745	36.016	14.381
51	Octacosane	C28H58	394.760	36.225	1.875
52	Nonacosane	C29H60	408.787	36.473	0.595
53	Triacontane	C30H62	422.813	37.222	
54	Hentriacontane	C31H64	436.840	38.010	
55	9,12-Octadecadienoic acid methyl ester	C19H34O2	294.470	39.696	4.663
56	9.12.15-Octadecatrienoic acid methyl ester	C19H32O2	292.456	39.800	5.113
57	3-ethyl-5-(2-ehylbutyl)-octadecane	C26H54O3	366.718	40.320	1.349
58	1-[1-Methyl-2-(octadecyloxy)ethoxy]octadecane	C39H80O	581.051	40.463	0.942
59	Tetratetracontane	C44H90	619.180	41.190	0.247
Total					99.198

Besides these three compounds, the next higher accumulating wax compounds those contained more than 2.0% of total wax compounds are 9.12.15- octadecatrienoic acid methyl ester, 9,12-octadecadienoic acid methyl ester, Docosane, Undecyl acetate, Trans-1,10-Dimethyl-*trans*-9-decalinol, 2,6,10,14-Tetramethylhexadecane and 2,5-Dipropyltetrahydrofuran having the levels of values of 5.113%,4.663%,3.178%, 3.163%, 2.839%,2.569% and 2.229%, respectively. There are some compounds those amounts ranged below 2.00% to 1.0%. They are Octacosane, Pentacosane, 5-propylnonane, 7,9-Dimethylhexadecane, 3-ethyl-5-(2-ehylbutyl)-octadecane, Ethyl tigalate, 10-Methyl-8-tetradecen-1-ol acetate and 6-Dodecanone having 1.875%, 1.669%, 1.526%,1.486%, 1.349%, 1.251%, 1.178% and 1.171%, respectively. The amount of 7-Hexylicosane wax compound was the lowest having 0.220% among the all compounds followed by Phytol acetate, Tetratetracontane, 2,4-Di-*tert*-butylphenol and 2,6,11,15-Tetramethylhexadecane containing the amount of 0.220%, 0.238%, 0.247%, 0.251% and 0.252%, respectively. Nine different wax compounds those were detected as a trace or no more amounts in the leaves of *Ziziphus nummularia* are 10-Methyl-8-tetradecen-1-ol acetate, 2.3.3-Trime thyl-2-(4-methylpentanoyl)-cyclopentanone, 2-Methyleicosane, Heneicosane, Hexadecanoic acid methyl ester, Ingol 12-acetate and Hentriacontane.

3.3. Variation of photosynthetic rate with temperature in *Ziziphus nummularia*

The photosynthetic rate varied from 0.70 to 7.70 μmol $CO_2/m^2/s$ against the studied temperature range of 15–55 °C (Fig. 3). At 15 °C the photosynthetic rate was 6.20 after which, with increase in temperature the photosynthetic rate tends to increased slightly up to 25 °C and it reached the highest level and after that a sharp variation was observed for next 5 °C increased temperature. After this point (30 °C), the photosynthetic rate remained constant until 40 °C and after further increase of temperature, the photosynthetic rate decreased sharply and it reached the lowest level of 0.70 at 55 °C.

3.4. Variation of transpiration rate with temperature in *Ziziphus nummularia*

The transpiration rate varies from 1.80 to 8.40 mmol $H_2O/m^2/s$ against a temperature range of 15–55 °C (Fig. 4). The rate of transpiration was 1.80 at 15 °C and with increase in temperature the transpiration rate increased very slowly up to 25 °C and then the rate slightly goes down at 30 °C. The transpiration rate was 8.40 at temperature 45 °C and the rate was reduced to 2.90 at 50 °C. At 55 °C the rate of transpiration was further reduced to 2.6.

3.5. Variation of quantum yield with temperature in *Ziziphus nummularia*

Much variation was observed in the quantum yield of photosystem II (F_v/F_m) against temperature in the leaves of *Ziziphus nummularia* from 0.63 to 0.80 F_v/F_m. (Fig. 5). At 15 °C, the quantum yield was 0.72 in the leaves of *Ziziphus nummularia* and after that with increased temperature from 15 to 20 °C, the quantum yield decreased to 0.63 F_v/F_m and with increase of next 5 °C the quantum yield increased sharply and it has reached its highest peak (0.80) at 25 °C. There after a decrease in quantum yield was noticed upto 35 °C and after which with increase in temperature quantum yield again started to raise further and remained constant until 50 °C. At 55 °C, the lowest quantum yield of 0.63 was observed.

4. Discussion

In the dry arid environments, an effective mechanism to regulate water loss at high temperatures is really significant for plants for their survival and maintenance of reproductive fitness. The stomata and the cuticle are considered as the main components of the plant regulatory mechanisms to preserve a favorable plant water status. The water stressed plants will close stomata to escape from dehydration and during the time of stomatal closure, the water permeability through the plant cuticle decides the lowest and unavoidable water loss (Schuster et al., 2017). Previous studies reported that *Ziziphus* species exhibit many drought tolerance properties such as effective photo protective mechanisms, osmotic regulation and stress dependent stomatal closure to delay water loss and avoid self-injury to thrive in arid environments (Clifford et al., 2002; Pareek, 2001).

In majority of the plants the wax compounds present in the cuticle contains a mixture of long straight chained hydrocarbons and their derivatives. Apart from this other components include branched hydrocarbons as well as cyclic compounds including secondary metabolites. But the variation in the cuticular wax composition is highly noticeable when emphasis lies on the chemical analysis of wax constituents among various plant species (Schuster, 2016). Analysis of the cuticular wax composition *of Ziziphus nummularia* revealed that it is qualitatively very much identical to that of some other arid plants. The main aliphatic class of wax components identified includes long-chain n-alkanes, alkanoic acids and 1° alkanols. Bush and McInerney (2015) reported that cuticle of plants in arid regions consists of components with comparatively longer average chain lengths than the plants studied from temperate zones. But it is very interesting to observe the chemistry of the cyclic component fraction of the cuticular waxes as they do not show much heterogeneity among the plants studied (Szakiel et al., 2012). Among the 62 compounds identified, 4-Hydroxy-cyclohexanone, Heptacosane and 2,7-Dimethyloctane-3,5-dione are the most dominant compounds. These three compounds represented 39.90% of the total wax compounds detected. It is documented that different types of wax compounds are commonly seen among some plant species (Aharoni et al., 2004; Szafranek and Synak, 2006) and at the same time some specific or a dominant classes of compounds are found in cuticular waxes of some plants (Ji and Jetter, 2008, Hansjakob et al., 2010, Oliveira and Salatino, 2000).

The transpiration rate varied significantly due to the variation of temperature in the leaves of *Ziziphus nummularia*. After 30 °C the transpiration rate started to increase rapidly and it has reached the highest value of 8.40 mmol $H_2O\ m^{-2}\ S^{-1}$ at 45 °C and after that transpiration rate decreased rapidly from 45 to 50 °C and there after a slight decrease at 55 °C. Yamori et al. (2014) regarded photosynthesis as an important and efficient temperature dependent phenomenon and stated that the response of photosynthetic traits to temperature is likely to reflect adaptations to the existing temperature. The present study shows that the leaves of *Ziziphus nummularia* can endure temperatures up to 40 °C without any damage to their photosynthetic capability. It is also mentionable that even at 55 °C though the photosynthetic rate was the lowest but photo-

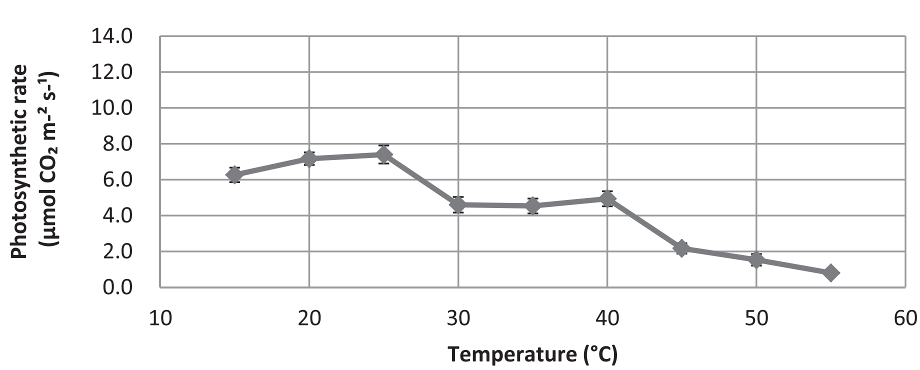


Fig. 3. Photosynthetic rate (μmol $CO_2\ m^{-2}\ s^{-1}$) in *Ziziphus nummularia*.

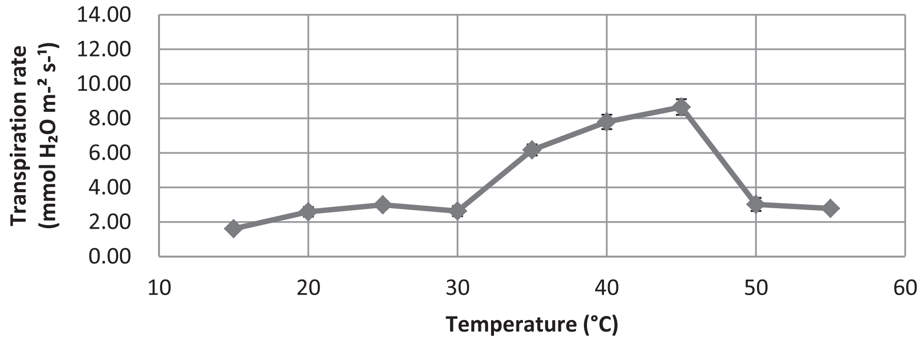


Fig. 4. Transpiration rate (mmol H_2O m^{-2} s^{-1}) in *Ziziphus nummularia.*

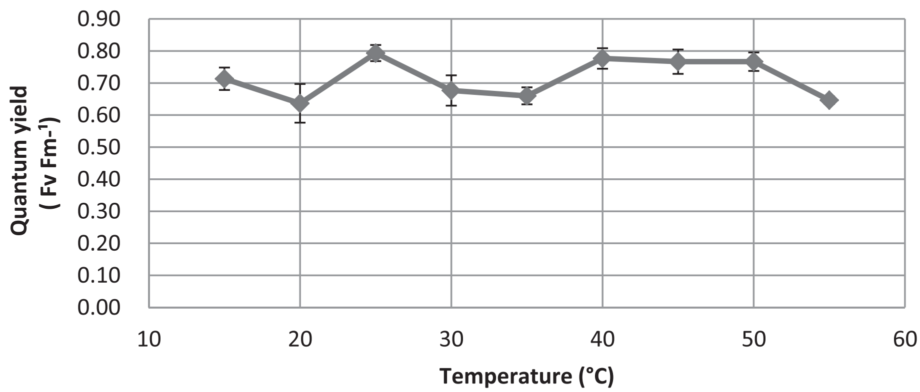


Fig. 5. Quantum yield (F_v/F_m) in *Ziziphus nummularia.*

synthetic rate did not stop. The ecophysiological studies in *Ziziphus nummlalaria* from the Indian arid zone have revealed that increase in transpiration enhanced the rate of net photosynthesis (Pandeya and Joshi, 1972). Chlorophyll fluorescence analysis has been used since a long time to demonstrate the functional variations of PSII photochemistry under temperature stress (Berry and Bjorkman, 1980). The variation of quantum yield was very closer at all the studied temperature from 15 to 55 °C. The quantum yield of photosystem II (F_v/F_m) of dark-adapted leaves remained constant at all the studied temperature from 15 to 35 °C and after that quantum yields tends to decrease slightly up to 55 °C which showed a strong effect of temperature on the heat stability of PSII in *Ziziphus nummularia* (Yamasaki et al., 2002). This validates the studies on some tropical plant species where a high temperature induced reduction of Fv/Fm occurred above 40 °C (Salvucci and Crafts-Brander, 2004). The present study results backs the view proposed by Gibson (1998) that saving water is not as central to desert plants as generally believed. Maximizing net photosynthesis and maintaining favourable leaf temperatures appear to be of equal or even higher importance.

Acknowledgment

This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award Number (12- ENV2564-02).

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