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Effects of hybridization and metaxenia on biochemical and molecular attributes of date palm (*Phoenix dactylifera* L.) Hillawi cultivar

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Abstract: Date palm (2n = 36) is a monocotyledonous, dioecious plant, which belongs to the family *Arecaceae*. Pollens are the primary source of diversity and improvement in quality, yield, ripening time, and size. Therefore, in this study, we investigated the differential behavior of nine pollen sources, collected from different locations, upon pollination with date palm female cultivar Hillawi. Metaxenia effect of pollen grains on the maternal tissues of fruits was carried out at khalal stage, whereas the seeds obtained from these fruits were planted in a nursery for hybrid confirmation. Biochemical characteristics; proximate analysis; individual sugar (sucrose, glucose, and fructose), total sugar, ascorbic acid, total soluble solid, and total phenolic contents; and 2,2-diphenyl-l-picrylhydrazyl (DPPH) and enzymatic activities (catalase, peroxidase, protease, and superoxide dismutase) were significantly variable. Dendrogram for biochemical parameters of cultivar Hillawi, created using Ward's method, revealed three clusters. Among the nine pollen sources, M8 was superior because it was positively associated with most of the variables. M7 shortened the ripening time, and its progeny exhibited the highest ascorbic acid content. Fruits developed from M8 pollination exhibited higher total sugar contents, protein, antioxidant and enzymatic activity, and had shortened ripening time. For more positive correlation of pollen parents, M8 was followed by M7, M3, M5, and M4. Strong positive correlation was found among most of the traits. Biochemical analyses revealed that these traits were suitable for metaxenia studies and breeding programs, as they exhibited quality and yield enhancement. The seedlings developed from these seeds, which were a result of controlled pollination, were identified as true date palm hybrids using 12 simple sequence repeats (SSRs) markers. Out of 12, only six primers clearly differentiated between true and ambiguous hybrids. Primer mpdCIR-10 identified the highest number of true hybrids 13 (81.25%). These findings will be useful for future studies.

Key words: *Phoenix dactylifera*, hybrid, biochemical and enzymes, sugars, SSR markers

1. Introduction

Date palm (*Phoenix dactylifera* L.) (2n = 36) is a monocotyledonous, dioecious plant, which belongs to the family Arecaceae. The Arecaceae family comprises 200 genera and 500 species (Dowson, 1982). Date palm fruits are nutritionally beneficial, especially in arid regions. Dates are also known as sugar palm (Al-Shahib and Marshall, 2003). Dates are composed of 44%–88% sugars, 2.3%–5.6% proteins, 6.4%–11.5% fibers, and small amount of fats, minerals, and vitamins (Al-sahib and Marshall, 2003). Moreover, date fruit is an important source of 23 amino acids that are not present in the majority of commonly consumed fruits (Al-Farsi et al., 2005). In date palms, pollination is important to ensure fertilization and fruit set because date palm exhibits metaxenia, which is the influence of pollen grains on developing maternal tissues (Maryam et al., 2015). Pollen grains have a direct impact on productivity, quality, yield, and fruit characteristics of cultivar Barhai (Abd-Elhaleem et al., 2020). Due to limited availability of date pollen, date palm growers use

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easily available pollen that affects productivity, fruit size, and quality with time (Osman et al., 1974; Moustafa et al., 2019). Due to metaxenia effects on date palm fruits, farmers prefer the most suitable pollen source that is positively correlated with quantity and quality of fruits. So far, many studies have been performed for seeking the most suitable pollen parents (Shahid et al., 2017; Outghouliast et al., 2020). Impact of pollen on nutritional properties of Medjool cultivar was assessed in Mexico (Salomón-Torres et al.,2018; Salomón-Torres et al., 2020)

Although antioxidants vary in their chemical structures, their function is to reduce oxidative damage. Antioxidants possess free-radical scavenging ability, which is important in case of injuries and diseases (Silva et al., 2007). Date palms are excellent source of antioxidants and exhibit 11,681–20,604 μmol oxygen radical absorbance capacity, which is equivalent to that of Trolox (g) (Al- Farsi et al., 2005). Antioxidants are not present in all phenolic compounds: in a previous study, 5000 phenolic compounds were investigated and only a few exhibited antioxidant activity (Robards et al., 1999; Karadeniz et al., 2005). In vegetables and fruits, phenolics inhibit the peroxidation of lipids (Rankin et al., 1993); thus, they are key antioxidants (Allaith, 2008). The stage of date palm fruits affects the total phenolic (PH) content, and PH content increases from kimri to khalal stage, whereas it decreases rapidly from rutab to tamar stage as the fruit ripens (Eid et al., 2011). Two date palm cultivars, namely Medjool and Deglet Nour, exhibited the maximum PH content, i.e. 572–661 GAE/100 g (FW) (Wu et al., 2004 a, b). Similar to PH content, enzymatic activity also changes with fruit maturation. Sudanese dates exhibit low polygalacturonase and cellulose activities at kimri stage (green stage), but the activities rapidly increase with fruit ripening (Mustafa et al., 2018). Moreover, the cellulose and pectinase collected at tamar stage are used in confectionary and beverage industries for the production of concentrated date syrup (Al-Hooti et al., 2002; El- Shornonby et al., 2009). In Deglet Nour extract, antioxidant activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were investigated, as these enzymes are related to damage prevention due to toxic elements with the dimethoade; GPx and SOD activity increased, whereas CAT activity decreased (Saafi et al., 2011; Awad et al., 2011a).

Borchani et al. (2010) reported content of reducing sugars (79.93–88.02 g/100 g) in eleven date palm cultivars. In Deglet Nour cultivar, the amount of sucrose (Suc; 54%) was higher than that of reducing sugars (26%–51%) (Mrabet et al., 2008). Sucrose content was highly negatively correlated with increasing levels of fructose (Fru) and glucose (Glu) (Amira et al., 2011; Vayalil, 2012). As the fruit ripens, protein content also increases (2.22–5.02 g/100 g FW), but in Deglet Nour cultivar, higher protein content was observed at khalal stage (Amira et al., 2011; Vayalil, 2012). Ascorbic acid (AA) or vitamin C, a water-soluble vitamin, plays a role in reversible oxidation processes. Generally, fruits and vegetables are considered good sources of AA, but date palm contains less amount of AA.

A previous study reported that AA content in Hillawi, Sayer, Khadrawi, and Zahdi cultivars of date were 3.56, 17.50, 3.20, and 2.40 mcg/100 g, respectively (Yousef et al., 1982). Rahemi (1998) reported that fruit quality and other fruit parameters of Shahani cultivar are influenced by pollen sources, except total soluble solids (TSS). The most essential targets of breeding trials are productivity enhancement, resistance against diseases, and insect pests. Different trials have been accomplished to confirm the variations in locally developed germplasm, but the long life cycle of date palm has made incorporation of these traits difficult (Al-Ruqaishi et al., 2008; Elshibli and Korpelainen 2009; Saker et al., 2006; Sedra et al., 1998; Zivdar et al., 2008). Repeated efforts in future may enhance important traits including adaptability against biotic and abiotic stresses and physiochemical characteristics in date cultivars. Hamwieh et al. (2010), using the AFLP markers, revealed that if F1 and F4 population are backcrossed, phenotypic and genotypic traits remain interrelated. Thus, the major objective of different breeding techniques is ensuring good quality and quantity of fruits with limited available resources (Carpenter and Ream, 1976; Zahid et al., 2022).

In Pakistan, immense diversity has been observed in male date palm plants (*Phoenix dactylifera* L.), but due to lack of breeding programs, they have not been properly investigated. Thus, it is essential to determine the best date palm pollen parents on the basis of yield and nutritional attributes. Therefore, in current study, nine date palm pollen sources were tested on Hillawi cultivar for metaxenia effects on biochemical attributes and resultant hybrid combinations could be evaluated for future breeding programs.

2. Material and methods

2.1 Pollen collection

Nine date palm pollen parents were collected after spathe breaking from three locations of Pakistan (Table 1) and dried under shade on newspaper and pollen powder was extracted and stored in paper bags.

2.2 Pollination

Three plants of cultivar Hillawi were selected in Horticultural Fruit Garden Sq. No. 32 of same age and vigor. Single plant was considered as a replication because spathes were selected on a tree for application of 9 male pollen sources. All the spathes were covered with paper bags to before and after pollination to ensure the true pollination. All the trees were pollinated manually by climbing over the tree and pollen grains were dusted

Table 1. Description of male and female along their collection site with progeny generated.

S. No.	Male	Collection site	Female	Collection site	Progeny (Hillawi)
1	M0	Open pollination	H1	Sq.#32 UAF	HM0
2	M1	Sq.#9 UAF	H2	Sq.#32 UAF	HM1
3	M2	Sq.#9 UAF	H3	Sq.#32 UAF	HM2
4	M3	Sq.#9 UAF			HM3
5	M4	Sq.#9 UAF			HM4
6	M5	D.I. Khan			HM5
7	M6	Uni. campus			HM6
8	M7	Sq.#32 UAF			HM7
9	M8	Sq.#32 UAF			HM8

over the spathes and bagged. The hands were washed thoroughly after every spathe pollination to avoid the possible mixing of pollen grains. Bags were removed from the bunches after four weeks of pollination for proper fruit growth.

2.3. Fruit collection and extraction for biochemical analysis

For biochemical analysis fruits were collected at khalal stage for extraction for biochemical analysis. The fruit flesh or respective treatment was grinded with 2 mL methanol and water (95% vlv). Extracts were filtered after centrifugation and stored at 4 °C until use.

2.4. Determination of different biochemical parameters

Protein content of fruit extract was estimated using Bradford method (Bradford, 1976). Using the method of Sadasivam and Manickam (1992) total soluble sugar of date fruits extract was measured. A digital refractometer was used for measure total soluble solids. Reducing and nonreducing sugars were measured with high-performance liquid chromatography (HPLC) methods as described by Booij et al. (1992). Total soluble solids were determined in fruits at khalal stage using the digital refractometer (ATAGO RX 5000, Japan Development Assistance). The scavenging abilities of fruits to 2,2-diphenyl-l-picrylhydrazyl (DPPH) stable radicals were measured to assess the antioxidant activity of date palm fruit extract. The DPPH assay was accomplished by following the method of Arem et al. (2012). Folin-Ciocalteu reagent was used for estimation of total phenolic contents and followed the method of Ainsworth and Gillespie (2007). For ascorbic acid assessment a sample mixture was prepared in a test tube using 900µL ddH_2O, 1 mL DCIP, 100 µL sample extract, 100 µL 0.1% meta H_3PO_4 and absorbance was documented at 520 nm.

2.5 Enzymatic studies

For sample extraction a phosphate buffer with pH 7 was used. Fruit samples were thoroughly washed with tap water and distill water to make them dirt-free. Samples were dried to remove excess moisture and cut into small pieces. One-gram fruit sample was mixed with 2 mL extraction buffer and mixture was finely grounded in pestle and mortar. This mixture was centrifuged at 4 °C for 10 min and supernatant was collected to remove particles. These filtrates were stored at 4 °C (Terras et al., 1993). Activity of catalase (CAT) was estimated with some modifications in method of Liu et al. (2009) and enzymes activities were expressed in terms of protein basis. Concentration of protein in crude extract was determined following the method of Bradford (1976). SOD activity was measured by estimating its ability to hinder the photoreduction of nitroblue tetrazolium (NBT) using the method of Giannopolitis and Ries (1977) and absorbance was measured at 560 nm with spectrophotometer. SOD unit is defined as "quantity of enzyme that is used to inhibit photo reduction of 50% of NBT". The method of Liu et al. (2009) was followed with some modifications for assessment of peroxidase activities (POD) and changes in absorbance of reaction solution was observed at 470 nm. One unit of POD activity is defined as "an absorbance change of 0.01 units per minute". Casein digestion assay was used to determine the protease activity as described by Drapeau (1974).

2.6 Data analysis

Experiment was laid out in RCBD design. XLSTAT was used for multivariate analysis for clustering the accessions. Statistical analysis was carried out by Statistics 8.1 and significant variations between means were also recorded by Duncan's multiple range test at $p < 0.05$.

3. Results

3.1 Variability among biochemical attributes of Hillawi cultivars pollinated with different male parents

The data collected for biochemical attributes were analyzed using Duncan's multiple range (DMR) test, and

the mean values were differentiated at 5% confidence interval (CI). The mean values of biochemical attributes are given in Table 2. The nine different pollen parents exhibited variation in total soluble sugar (TS) content at khalal stage. Pollen parent M8 (33.33 ± 0.33%) exhibited the highest sugar content when pollinated with female cultivar Hillawi, whereas the lowest amount of sugar was observed with pollen parent M0 (19.00 ± 0.58%). Female parent possessed the highest TS content when pollinated with M8 (63.24 ± 0.89 µg/mL), followed by that with M7 (53.61 ± 1.67 µg/mL), which was at par with that by M2, whereas minimum TS content was observed with pollen parent M0 (24.58 ± 0.66 µg/mL). We observed the highest AA content with M7 pollen parent (6.20 ± 0.51 mg/100 g) compared to that with other male parents. Data presented in Table 2 shows that all pollen grains had a significant effect on PH content of cultivar Hillawi, with the maximum effect observed with M8 (491.20 ± 4.59 mg GAE/100 g), followed by that with M7 (389.48 ± 15.46 mg GAE/100 g) and M5 (365.02 ± 3.10 mg GAE/100 g), whereas the lowest phenolic content was observed in case of open pollination (190.19 ± 3.13 mg GAE/100 g).

TS content was significantly low in open pollinated plants (7.77 ± 0.15gh), whereas it was the highest with pollen parent M8 (15.13 ± 0.08a), and the difference with other parents was not significant. Moreover, plants with pollen parent M8 produced the highest protein content (7.45 ± 0.08 µg/mL), followed by those with pollen parents M7, M1, M3, and M5 (in descending order). Reducing sugar content was the highest with M8 and M6 pollen parents (18.00 ± 0.58% and 14.00 ± 0.58%), respectively. Among the different pollen sources, the highest Glu content (26 ± 0.58%) was observed with M8, followed by that with M3 (24.67 ± 0.88b) and M6 (23 ± 0.58c), whereas the lowest Glu content was observed with open pollination M0 (12 ± 0.58j). The highest Fru content was observed with M8 and M3 pollen parents (20.00 ± 0.58%), whereas the lowest Fru content was observed with M0 (9.00 ± 0.58k). POD activity was the highest with M5 (1.517 ± 0.009 IU/mL protein), followed by activities in case of M8 and M3 pollen parents, which were significantly similar (1.41 ± 0.015 and 1.42 ± 0.012 IU/mL protein, respectively); fruits with M6, M4, and M7 pollen parents exhibited similar POD activities, i.e. 1.343 ± 0.009, 1.213 ± 0.07 and 1.14 ± 0.017fg IU/mL protein, respectively, whereas M0 exhibited low (0.222 ± 0.014 IU/mL protein) POD activity at khalal stage. Hillawi cultivar pollinated with pollen parent M8 exhibited the highest catalase (CAT) activity (2.21 ± 0.150 IU/mL protein), followed by that with M6 (2.03 ± 0.024 IU/mL protein), M3 (1.78 ± 0.033 IU/mL protein), and M4 (1.71 ± 0.219 IU/mL protein), whereas the lowest activity was recorded with M0 (0.26 ± 0.069 IU/mL protein).

SOD activity was statistically analyzed at khalal stage in date palm fruits, and analysis of variance revealed highly significant differences in SOD activity of the pollen parents. Fruits with M8 pollen parents exhibited the highest and significant SOD activity (1300.67 ± 5.81 IU/mL protein), whereas fruits with M0 exhibited the lowest activity (1056 ± 5.49 IU/mL protein), which was at par with that with M1 (1090.67 ± 5.81 IU/mL protein). All pollen parents exhibited a highly significant effect on protease activity (PRO). Male parents were grouped into eight statistically distinct groups with M8 at the top. Maximum PRO activity in Hillawi cultivar was observed when it was pollinated with M8 (25.11 ± 0.49 IU/mL protein), followed by that with M6 (265.31 ± 3.74 IU/mL protein) and M3 (255.86 ± 1.82 IU/mL protein), and the lowest was observed with M0 (114.23 ± 1.69 IU/mL protein).

3.2 Correlation studies of biochemical attributes

Pearson correlation analysis revealed strong positive correlations among 13 biochemical traits in all combinations of the Hillawi cultivar (Figure 1). TS content was strongly correlated ($p < 0.01$) with PH content ($r = 0.81^{**}$), TSS content ($r = 0.89^{**}$), and Suc content ($r = 0.84^{**}$). Individual sugar content, i.e. Fru ($r = 0.76^{*}$) and Glu ($r = 0.73^{*}$) contents, PRO activity ($r = 0.71^{*}$), and total protein content (PR, $r = 0.78^{*}$) exhibited good correlation ($p < 0.05$) with TS content (Figure 1). PR content exhibited a strong positive correlation ($p < 0.01$) with PH content ($r = 0.84^{**}$). PR content also exhibited significant ($p < 0.05$) and positive association with TSS content ($r = 0.78^{*}$) and PRO activity ($r = 0.70^{*}$), whereas it exhibited weak negative correlation with AA content ($r = 0.187$). Moreover, individual sugars exhibited strong positive correlation ($p < 0.01$) among each other, i.e. Glu with Fru ($r = 0.79^{*}$), Fru with Suc ($r = 0.72^{*}$), and Glu with Suc ($r = 0.71^{*}$). Strong positive correlation was observed for PRO activity with Fru, Suc, and Glu contents ($r = 0.88^{**}$, $r = 0.82^{**}$, and $r = 0.77^{*}$, respectively). Similar to sugars, enzymes exhibited a strong positive correlation with each other, i.e. PRO was strongly positively correlated with POD ($r = 0.92^{**}$), followed by CAT ($r = 0.91^{**}$), whereas it was moderately correlated ($p > 0.05$) with SOD ($r = 0.588$). 1,1-diphenyl-2-picrylhydrazyl (DPPH) exhibited highly significant association with Suc content ($r = 0.81^{**}$). We also observed negative correlations for some biochemical traits. AA exhibited weak negative correlation with TS content ($r = -0.23$), PR content ($r = -0.19$), PH content ($r = -0.29$), SOD activity ($r = -0.21$), CAT activity ($r = -0.11$), Glu content ($r = -0.26$), Suc content ($r = -0.26$), and TSS content ($r = -0.39$).

3.3 Principal component analysis (PCA) of biochemical attributes

The PCA plot based on biochemical analysis of fruits of various pollen parents revealed that the different combinations were scattered among all four planes (Figure

Table 2. Metaxenia effects of nine different pollen parents on biochemical attributes of cultivar Hillawi.

Traits	M0	M1	M2	M3	M4	M5	M6	M7	M8
TS	19 ± 0.58gh	26.33 ± 0.33c	30.67 ± 0.33b	30 ± 0.58b	23.67 ± 0.33d	23.33 ± 0.67de	24 ± 0.00d	29.33 ± 0.33b	33.33 ± 0.33a
DPPH	24.58 ± 0.66j	41.94 ± 0.65g	52.04 ± 0.73f	38.43 ± 0.51h	27.34 ± 0.58j	33.41 ± 0.58i	42.03 ± 0.55g	53.61 ± 1.67f	63.24 ± 0.89cd
AA	2.73 ± 1.27d	4.93 ± 1bc	4.33 ± 0.09bc	4.5 ± 0.26c	5.1 ± 0.55bc	5.4 ± 0.25ac	4.7 ± 0.17abc	5.47 ± 0.44a	3.57 ± 1.37c
PH	190.19 ± 3.13n	230.48 ± 4.61l	346.24 ± 0.62e	322.81 ± 0.88f	283.02 ± 2.76i	365.02 ± 3.10d	243.93 ± 2.79k	389.43 ± 5.46c	491.2 ± 4.59a
TSS	7.77 ± 0.15gh	9.33 ± 0.18efg	10.99 ± 0.01cf	13.69 ± 0.26ab	8.8 ± 0.47fgh	8.95 ± 0.03fgh	9.88 ± 0.18efg	12.92 ± 0.2bc	15.13 ± 0.08a
PR	3.2 ± 0.22d	6.02 ± 0.36bc	4.62 ± 0.3c	5.61 ± 1.04bc	4.81 ± 0.07bc	5.28 ± 0.11bc	4.62 ± 0.11c	5.87 ± 0.42b	7.75 ± 0.69a
Suc%	8 ± 0.58hi	11 ± 0.58ef	15 ± 0.58b	12 ± 0.58de	10.33 ± 0.33f	10 ± 0.58fg	14 ± 0.58bc	15 ± 0.58b	18 ± 0.58a
Glu%	12 ± 0.58j	14 ± 0.58i	20 ± 0.58e	24.67 ± 0.88b	16 ± 0.58g	15 ± 0.58h	23 ± 0.58c	17 ± 0.0f	26 ± 0.58a
Fru	9 ± 0.58k	17 ± 0.58cd	19 ± 0.58ab	20 ± 0.58a	16 ± 0.58de	18 ± 0.58bc	19.33 ± 0.67ab	15.33 ± 0.67dg	20 ± 0.58a
POD	0.218 ± 0.024j	0.901 ± 0.023h	1.127 ± 0.009g	1.42 ± 0.012cd	1.213 ± 0.07ef	1.517 ± 0.009ab	1.343 ± 0.009d	1.14 ± 0.017fg	1.41 ± 0.015cd
CAT	0.26 ± 0.069j	0.91 ± 0.026i	1.28 ± 0.049gh	1.78 ± 0.033cde	1.71 ± 0.219def	1.13 ± 0.015hi	2.03 ± 0.024bc	1.45 ± 0.076fg	2.21 ± 0.15b
SOD	1056 ± 5.49k	1090.67 ± 5.81j	1181.33 ± 1.33ef	1110.67 ± 2.96i	1191 ± 1.00e	1251.67 ± 1.67c	1151.67 ± 1.67g	1130.67 ± 3.48h	1300.67 ± 5.81a
PRO	114.23 ± 1.59i	204.08 ± 1.40h	225.46 ± 0.46f	255.86 ± 1.82c	204.16 ± 0.78h	237.9 ± 2.50e	265.31 ± 3.74b	246.62 ± 1.91d	272.02 ± 5.09a

Total sugars (TS), 1,1-diphenyl-2-picrylhydrazyl (DPPH, µg/mL), ascorbic acid (AA, mg/100g), total phenolic contents (PH, mg GAE/100g), total soluble solids (TSS, °Brix), soluble protein (PR, µg/mL), sucrose (Suc, %), glucose (Glu, %), fructose (Fru), peroxidase (POD, IU/mL protein), catalase (CAT, IU/mL protein), superoxide dismutase (SOD, IU/mL protein), and protease (PRO, IU/mL protein).

Figure 1. Correlation matrix showing the relation of thirteen biochemical traits in cultivar Hillawi. Total sugars (TS), 1,1-diphenyl-2-picrylhydrazyl (DPPH, µg/mL), ascorbic acid (AA, mg/100g), total phenolic contents (PH, mg GAE/100g), total soluble solids (TSS, °Brix), soluble protein (PR, µg/mL), sucrose (Suc, %), glucose (Glu, %), fructose (Fru), peroxidase (POD, IU/mL protein), catalase (CAT, IU/mL protein), superoxide dismutase (SOD, IU/mL protein), and protease (PRO, IU/mL protein). * and ** represent significance at $p < 0.05$ and 0.01, respectively.

2). HM1, HM4, and HM5 were present in the first quadrate and formed a separate group, whereas HM0 was present in the second quadrate. Similarly, HM8 was the most divergent compared to the other combinations and clustered with HM7 and HM2 within the same quadrate (third). HM3 and HM6 were present in the fourth quadrate and formed another group; thus, these groups were closely related to each other but were different from HM8. In the PCA biplot, scattering of all pollen parents along the biochemical traits indicated diversity, with respect to each pollen parent. The combination HM8 was sweeter, as it was positively correlated with Suc, Glu, PH, TS, and protein content. SOD activity and DPPH were the highest in HM3. HM7 and HM2 exhibited a strong association with Glu content; however, HM3 exhibited strong association with DPPH and SOD activity. The highest POD activity was observed in HM6, and HM5 exhibited association with AA. However, HM0 grouped irrespective of any biochemical activity, which indicates that this is not very important (Figure 2).

3.4 Cluster analysis

The dendrogram shows three clusters that are phenotypically correlated with nine different combinations of their respective parents (Figure 3). HM0 formed a separate cluster, i.e. cluster 1, as it was the most divergent among all genotypes. Cluster 2 comprised seven genotypes

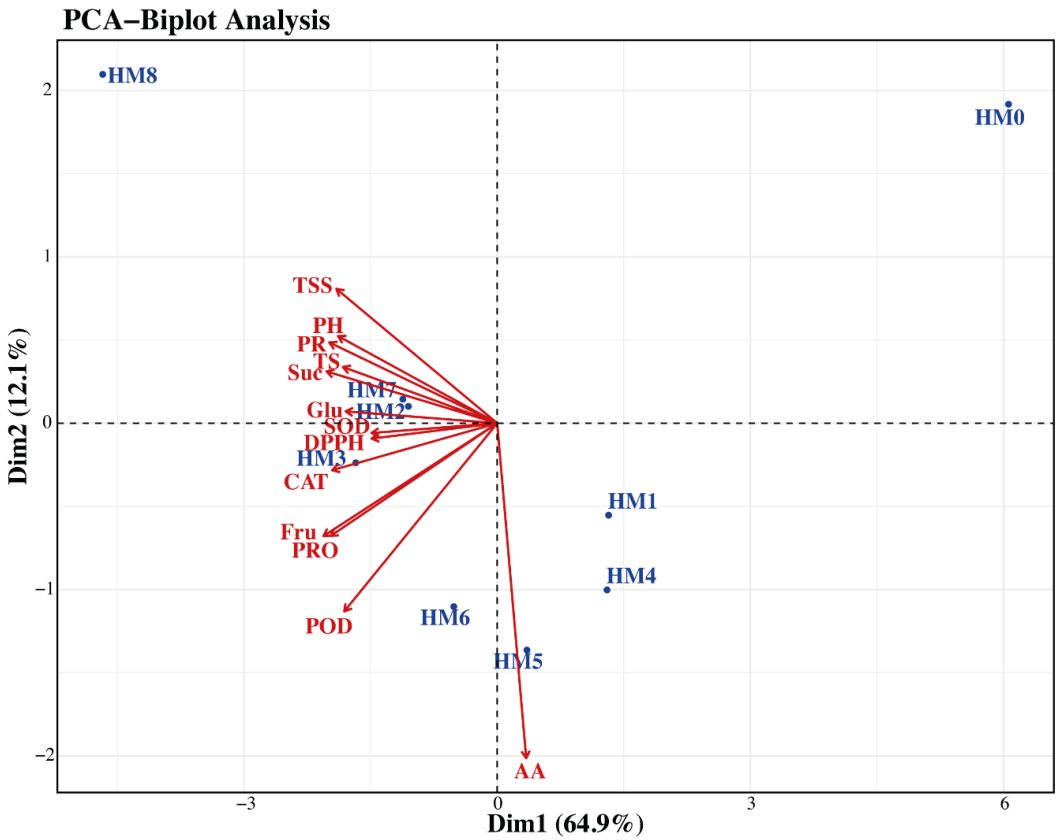

Figure 2. Principal component analysis (PCA) plot based on biochemical traits of cultivar Hillawi. PCA biplot showed the variation in 9 different cultivars in relation to biochemical traits. Total sugars (TS), 1,1-diphenyl-2-picrylhydrazyl (DPPH, µg/mL), ascorbic acid (AA, mg/100g), total phenolic contents (TP, mg GAE/100g), total soluble solids (TSS, °Brix), soluble protein (PR, µg/mL), sucrose (Suc, %), glucose (Glu, %), fructose (Fru), peroxidase (POD, IU/mL protein), catalase (CAT, IU/mL protein), superoxide dismutase (SOD, IU/mL protein), and protease (PRO, IU/mL protein).

and formed three subgroups. HM3 and HM7 were closely related and clustered under the same subgroup; similarly, HM5, HM4, and HM2 showed close association and formed a subgroup. HM1 and HM6 were closely linked and formed the third subgroup, but all three subgroups were closely linked (Figure 3). Similarly, cluster 3, the last cluster, contained only one genotype (HM8).

3.5 Hybridization in date palm for germplasm enhancement

Simple sequence repeats (SSRs) were used for the identification of date palm progeny developed from the pollination of nine different pollen parents with cultivar Hillawi. True hybrids were identified using 12 SSR primers, and out of 12, only six primers could amplify a 125–500-bp fragment. These six SSR primers produced 66 loci with an average of 11 loci per primer. Major allele frequency varied from 0.5 to 0.63 with primers mpdCIR015 and DP159. Hybrid genotypes were moderately diverse, with diversity being 0.31–0.54 (Table 3).

The date palm hybrid identification microsatellite primer mpdCIR-10 determined true hybrids developed from the selected male pollen parent and cultivar Hillawi (Figure 4). Hybrids 12 to 19 (H1M1, H1M2, HM3, HM4, HM5, HM6, HM7, and HM8) were combinations of Hillawi with eight different selected male plants. A monomorphic locus of 210 bp, which was identified in female plants, was found in all hybrids, whereas the male fragments differed. Hybrid 12 (H1M1) shared one locus from the female parent (210 bp) and two loci (220 bp and 290 bp) from male parent M1, with a unique locus of 250 bp. In hybrid 13 (HM2), female parent contributed a monomorphic locus of 210 bp, whereas male parent M2 contributed two loci of 240 bp and 320 bp (Figure 4). Hybrid 14 was the true hybrid of Hillawi cultivar and male parent M3, as the female and male parents contributed a single locus of 210 bp and two loci of 225 bp and 320 bp, respectively, but a unique 160-bp fragment was present in the progeny that was absent in both parents. Hybrid 17 (HM6) was

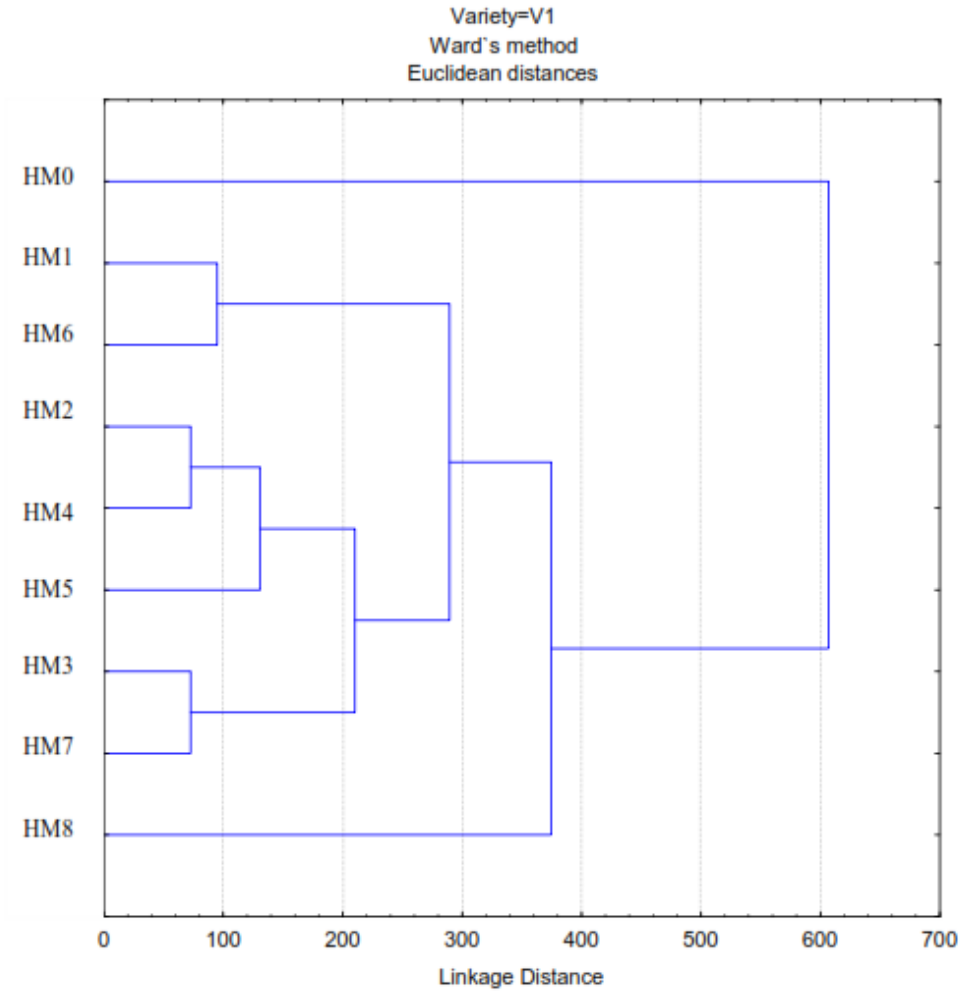

Figure 3. Cluster analysis of pollen parents based on biochemical analysis in cultivar Hillawi.

Table 3. SSR primers detail and summary of genetic diversity information by locus in date palm parents and progeny cv. Hillawi used for hybrid confirmation.

Sr. No	P. code	AS	MAF	G. No	GD	HZ	PIC
1	mPdCIR010	145-340	0.51	18	0.58	0.31	0.50
2	mPdCIR015	140-190	0.5	4	0.54	0.73	0.46
3	mPdCIR016	100-220	0.55	10	0.51	0.33	0.40
4	mPdCIR025	210-320	0.62	10	0.48	0.36	0.42
5	mPdCIR057	175-500	0.58	17	0.5	0.38	0.42
6	DP159	125-175	0.63	7	0.46	0.54	0.37
		Total	3.39	66	3.07	2.65	2.58
		Mean	0.57	11.00	0.51	0.44	0.43

P. code = primer code, AS = allele size, MAF = major allele frequency, G. No = genotype number, GD = genetic diversity, HZ = heterozygosity, PIC = polymorphic information contents.

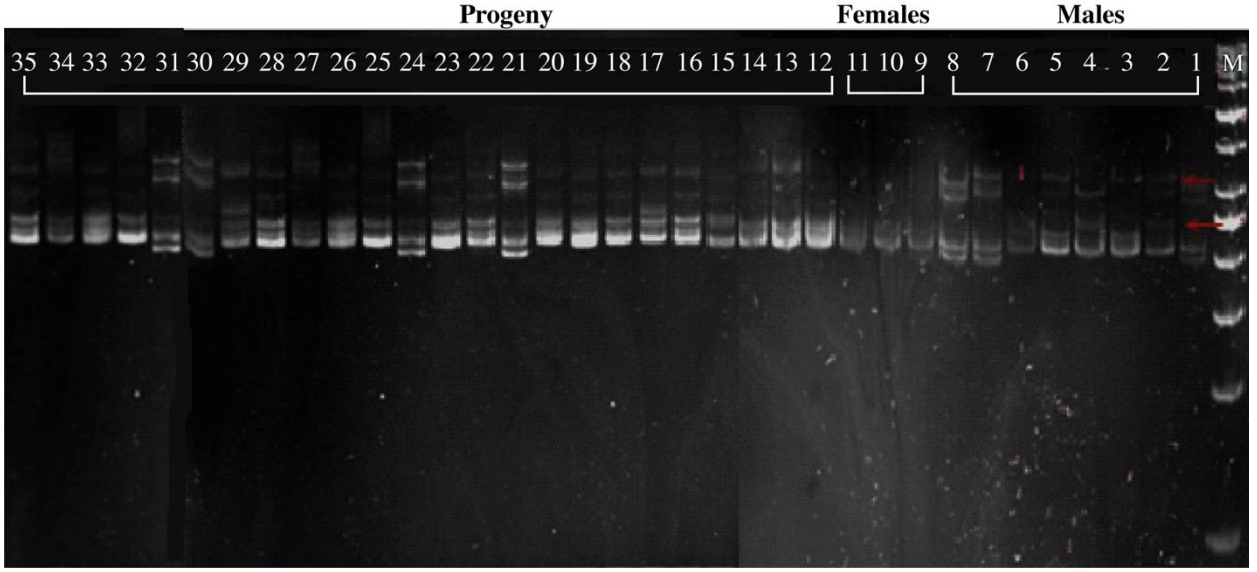

Figure 4: Hybrid identification of 30 date palm genotypes including eight males (1–8) and three females (9–11) along their progeny (15–30) with SSR primers mpdCIR25. M is 50 bp ladder (Fermentas, USA).

the true hybrid of Hillawi and male parent M6, as it contained a homozygous locus (210 bp) from the female parent and another homozygous locus from (225 bp) the male parent M6. Similarly, hybrid HM7 obtained three loci (190 bp, 300 bp, and 320 bp) from the male parent M7 and a single homozygous locus of 210 bp from the female parent. HM8 obtained two fragments (280 bp and 310 bp) from the male parent M8 and a single locus of 210 bp from the female parent. Thus, the abovementioned hybrids were the true hybrids of their respective parents.

4. Discussion

Some biochemical variables are preferred to determine the efficacy of different pollen parents with female date palm cultivar Hillawi. For genotype characterization, these variables are significant tools (Rodriguez et al., 2008). Date palm yield and fruit quality are the most important and targeted traits for breeders due to their economic importance. TS content observed in cultivar Hillawi at khalal stage was significantly higher when pollinated with M8 and the lowest when openly pollinated. Osman et al. (2002) used three different pollen sources, namely, Ghiza, Rashid, and Aswan, to pollinate cultivar Hillawi and observed difference in TS content with all three pollen sources. Hamwieh et al. (2010) partially confirmed our findings, as they reported differences in TS content in Seewy cultivar (32.20%, 34.60%, and 33.10%) when pollinated with three different pollen sources (Asswan, El-Fayoum, and El-Sharkia). Al-Najam et al. (2021) investigated the xenic and metaxenia effects of in vitro-derived female plants of cultivar Barhee and observed variations in TS content. Furthermore, the quince pollen (*Cydonia oblonga*) dramatically improved the fruit weight, fruit hardness, vitamin C content, sugar-acid ratio, total phenolics and total flavonoids of apple cultivars, which resulted in fruit quality improvement (Zhang et al., 2019).

TSS content was 7.77–15.10 ° Brix in cultivar Hillawi. Our findings are similar to those reported by Osman et al. (2010) that pollen from diverse areas influenced TSS content and age improvement. El-Hamady et al. (2010) pollinated the "Hayany" date palm cv. with two pollen sources and reported that it gave higher value (28.5%) at khalal stage, when pollinated with M1 compared to M2 pollen grains. These results are also in accordance with those reported by AI-Obeed and Abdul-Rahman (2002), Awad and Al-Qurashi (2012), and Farag et al. (2012), but they are contradictory to the results reported by Rahemi (1998) that TSS content remained the same irrespective of the pollen source; however, the present study validated that pollen source influences TSS content in date palm. These observed variations in TSS could be due to genetic variations, spathe characteristics, vigor (Nasir et al., 1986), and tree age (Ibrahim et al., 1994).

Date palm flesh is a minor source of proteins, but in our study, the protein content was 3.03–7.55 µg/mL when Hillawi cultivar was pollinated with male parents M0 and M8. Amira et al. (2011) and Vayalil (2012) also investigated the protein content (2.22–5.02 g/100 g FW) in date palm fruits. Similar protein level patterns were observed for date palm fruits from Libya (Ishurd et al., 2004) and United Arab Emirates (Ismail et al., 2008), but the range was relatively higher at khalal stage than at other stages, which could be due to enzymatic activities. Vitamin

C content was the highest at early fruit developmental stages but gradually decreased, which could be due to the inability of the fruit to accumulate AA or dilution of AA during ripening (Awad, 2011). The study by Yousaf et al. (1982) also reported similar results and determined AA content in Halawi (3.56 mcg/100 g), Zahdi (2.40 mcg/100 g), Khadrawy (3.20 mcg/100 g), and Sayer (17.50 mcg/100 g) cultivars.

PH content in cultivar Hillawi was 318.17 mg GAE/mg, which varied from 201.17 mg GAE/mg to 485.70 mg GAE/mg with different male parents. These findings are similar to those reported by Saafi et al. (2009); they determined PH content (210.17 mg GAE/mg and 485.70 mg GAE/mg FW) in two cultivars of date palm, i.e. Kentichi and Allig, respectively. The results of current study are in line with Shahsavar and Shahhosseini (2022), who found that total phenolics and sugar contents were varied due to metaxenia effect of pollen source in date palm. Phenolic compounds are an important component of date palm fruits, and different cultivars retain different concentrations of antioxidants (Al- Farsi et al., 2007a). Maximum amount of PH is found in date palm at khalal stage (Eid et al., 2011); however, variation in PH content was reported by Thabet et al. (2009), Chaira et al. (2009), and Eid et al. (2011). PH content in date palm fruits was similar to or higher than that of other common fruits, i.e. 4.1–12.4 mg GA/100 g in oil palm (Czerniak et al., 2011), 228 mg/100 g FW in raspberries (Proteggente et al., 2002), 28 mg/100 g in papaya (Lim et al., 2007), and 51 mg/100 g and 48 mg/100 g FW in banana and apple, respectively (Lim et al., 2007).

AA content gradually increased at early developmental stages of the fruits, but it decreased with maturity, with AA content being 2.73 and 5.47 mg/100 g in cultivar Hillawi when pollinated with M0 and M7, respectively, and this finding is in accordance with the results reported by Yousaf et al. (1982), as they reported different AA content in four date palm cultivars Zahdi Halawi, Khadrawy, and Sayer, i.e. 2.40 mcg/100 g, 3.56 mcg/100 g, 3.20 mcg/100 g, and 17.50 mcg/100 g, respectively.

The comparison of mean enzymatic activities revealed that M8 was the best pollen source. Enzymatic activities varied widely with pollen source; CAT, POD, SOD, and PRO activities were 0.30–1.98 IU/mL, 1075.73–1304.50 IU/mL, and 114.34–268.17 IU/mL, respectively. Enzyme activities improve with maturity; however, the increase is rapid as the fruit ripens. In the current study, variation in enzymatic activities could be due to the female spathe that was covered with a paper bag to reduce astray pollen contamination. Antioxidant activity and PH content were strongly correlated. In date palm, positive correlation has been observed between PH content and antioxidant activity (Mansouri et al., 2005; Al-Turkey et al., 2010), which is similar to that in other fruits (Javanmardi et al., 2003; Leontiwics et al., 2003). All enzymes and sugars showed strong positive correlation with each other; however, some differences were observed, which could be due to different reaction conditions, analytical techniques, mechanisms, and other factors, including location, temperature, soil, and sunlight.

The results of the current study revealed the significance of SSR for hybrid characterization. Although SSRs are highly polymorphic, reproducible, and precise, few microsatellite markers have also been observed (Hamwieh et al., 2010). The importance of microsatellite markers has been broadly discussed for hybrid evaluation and genetic purity assessment in other crops, e.g., pear (Yamamoto, 2001), peach (Wang et al., 2002), and tomato (Liu et al., 2007). Recently, SSR markers have been exploited for hybrid analysis of date palm progeny. The current study revealed that SSRs are highly polymorphic, containing vast numbers of alleles. Our findings are supported by those of Elmeer and Mattat (2012) who reported 8.86 alleles per locus, which is close to 8.83 alleles per locus out of total 53 loci but was higher than those reported by Ahmad and Al-Qaradawi (2009), as they investigated 15 date palm cultivars and recorded a mean of 4 alleles per locus. This notable difference might be due to genotype variations and a higher number of microsatellite (16) markers used in this experiment. Six SSR primers were able to amplify a 125–340 bp locus, which is in accordance with the results reported by Ahmed and Al-Qaradawi (2009), i.e. amplification of a 100–300-bp locus. MpdCIR93 and mpdCIR10 exhibited genetic diversity, with 0.38 to 0.58 per locus among parents and the hybrid progeny. Observed level of genetic diversity is less than that for Sudanese date palm germplasm (0.70) (Zehdi et al., 2004) and Tunisian date palm (0.853) (Elshibli and Koropelainen, 2007). Variations in results could be due to a shared or narrow genetic pool, as the progeny was developed via hybridization; however, divergence has also been found among some progeny, which could be due to some mutational events that occurred during selection.

5. Conclusion

Conventional date palm breeding is a time consuming and laborious process. Due to metaxenia effect, yield and quality attributes of date palm fruit could be improved. The results based on biochemical attributes of date palm fruit depicted that three pollen sources influenced notable metaxenia effects on yield and quality parameters. Potential hybrid combinations of Hillawi cultivar were developed with nine different pollen sources. These new hybrid combinations can be evaluated for the selection of better hybrids in future breeding programs. The results depicted that M8 was found to be the best pollen source, as it not only shortened the ripening time but positively correlated with other biochemical traits. Additionally, SSR markers were identified in this study, which can be used in future studies for the identification of hybrids.

Conflict of interest
The authors declare that they have no conflict of interest.

Contribution of authors
Maryam, Ishtiaq Ahmad and Muhammad Nafees designed the study. Maryam, Rashid Iqbal, and Sana Fatima performed the experiments and analysis. Maryam, Ishtiaq Ahmad, Muhammad Nafees, and Muhammad Uzair wrote the original draft. Sajid Fiaz, Hayat Ali Alafari, Dalal S. Alshaya, and Muhammad Jafar Jaskani guided during the whole study. Muhammad Jafar Jaskani supervised the whole study. Sajid Fiaz, Kotb Attia, Arif M Mohamed and Hayat Ali Alafari provided technical expertise to improve the article and helped in funding acquisition. All authors reviewed and edited the manuscript.

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Data availability statement
All the data related to this study is presented in the main text.

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Supplementary data

Table S1. Primers details.

Sr. No	Primer code	Primer forward sequence	Primer Reverse sequence	Exp. Size	Motif repeat	Tmp (0C)	status of amplification
1	mPdCIR010	ACCCCGGACGTGAGGTG	CGTCGATCTCCTCCTTTGTCTC	114-236	(GA)22	55.9	**
2	mPdCIR015	AGCTGGCTCCTCCCTTCTTA	GCTCGGTTGGACTTGTTCT	104-150	(GA)15	51.6	**
3	mPdCIR016	AGCGGGAAATGAAAAGGTAT	ATGAAAACGTGCCAAATGTC	104-198	(GA)14	51.7	**
4	mPdCIR025	GCACGAGAAGGCTTATAGT	CCCCTCATTAGGATTCTAC	192-244	(GA)22	49.3	**
5	mPdCIR057	AAGCAGCAGCCCTTCCGTAG	GTTCTCACTCGCCCAAAAATAC	214-284	(GA)20	55.4	**
6	DP159	AGCTCCAATTTGCTGCAGAG	GCTGACCTGGAGTCCAAAAC	156-172	(TC)27	54.3	**