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Pollen viability in natural populations of *Thymus capitatus* the south Al-Jabal Al-Akhdar area, Cyrenaica, Libya.

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Abstract: Viable pollen plays a crucial role in the process of seed formation and acts as the principal mechanism for gene transfer in outcrossing species such as thyme (Thymus capitatus). There is a lack of information on the pollen viability of natural populations of Libyan thyme that grow in the south El-Jabal El-Akhdar area. This research estimated pollen viability using acetocarmine staining method. It was possible to distinguish between viable pollen (non-aborted) and non-viable (aborted) pollen using acetocarmine stain. The viability of pollen percentage in T. capitatus accessions was found to be significantly different. The viable pollen recorded as highest was obtained from the white-flowered population (67.61%) followed by dotted white-flowered population (57.24%), while the lowest viable pollen was obtained from mosaic- lowered population (29.86%). The remaining two populations, violet-flowered and purple-flowered showed 55.73% and 42.38% respectively as pollen viability percentage. Variation was also observed in the pollen viability among different collection periods. These results suggested that pollen viability may be under both environmental and genetic control.

Keywords: Pollen viability; acetocarmine staining method; *Thymus capitatus*; El-Jabal El-Akhdar; Libya.

حيوية حبوب اللقاح في المجتمعات الطبيعية لنبات الزعتر البري *Thymus capitatus* في منطقة جنوب الجبل الأخضر، برقة، ليبيا.

المستخلص : حبوب اللقاح الخصبة والحية ضرورية لتكوين البذور، وتعمل بمثابة وسيلة أساسية لتدفق الجينات في الأنواع النباتية مفتوحة التلقيح مثل نبات الزعتر(.*Thymus capitatus* L). هناك نقص في المعلومات عن خصوبة حبوب اللقاح (حيوية حبوب اللقاح) في العشائر الطبيعية للزعتر البري، والذي ينتشر بصورة طبيعية في منطقة جنوب الجبل الأخضر. في هذا البحث قدرت حيوية حبوب اللقاح باستخدام صبغة الأسيتوكارمين. وقد بينت الدراسة فاعلية هذه الطريقة، فقد كان من الممكن التمييز بين حبوب اللقاح الخصبة (الحية) وحبوب اللقاح غير الخصبة (المجهضة). اشارت النتائج إلى أن الاختلافات في نسب حيوية حبوب اللقاح في العشائر الطبيعية للزعتر الليبي (.*Thymus capitatus* L) كانت ذات فروق معنوية. فقد سجلت عشيرة النباتات ذات الازهار البيضاء أعلى نسبة لحبوب اللقاح الخصبة (67.61%)، تليها مباشرة عشيرة النباتات ذات الازهار البيضاء المنقطة (57.24%)، بالمقابل سجلت عشيرة النباتات ذات الازهار المبرقشة أقل نسبة لحبوب اللقاح الخصبة (29.86%). العشيرتين: عشيرة النباتات بنفسجية الازهار وعشيرة النباتات ارجوانية الازهار سجلت النسب 55.73% و 42.36% على التوالي. أيضاً بينت النتائج أن هناك تباين في حيوية حبوب اللقاح بين مختلف فترات التجميع. هذه النتائج تشير بأن حيوية حبوب اللقاح في عشائر الزعتر تخضع لفعل العوامل البيئية والعوامل الوراثية.

الكلمات المفتاحية: حيوية حبوب اللقاح، طريقة اختبار صبغة أسيتوكارمن، الزعتر البري، الجبل الاخضر - ليبيا.

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INTRODUCTION

Effective pollination is essential for the successful development of fruits and seeds in the majority of plant species. Understanding pollen biology, particularly aspects related to pollen quality, is crucial for implementing strategies aimed at enhancing agricultural productivity (Shivanna, 2003). Pollen quality plays a significant role in the overall fitness of plants and marks a vital phase in their life cycle. It is commonly associated with pollen viability, which refers to the percentage of pollen grains that remain viable (Stanley and Linskens, 1974; Heslop-Harrison *et al.*, 1984).

The extensive body of literature concerning pollination ecology and pollen biology reveals a considerable diversity in the terminology and definitions associated with the functional capabilities of pollen. Viability is commonly defined as the "capacity to live, grow, germinate, or develop" (Lincoln *et al.*, 1982). Additionally, the concept of viability has been employed to refer to pollen grains that can germinate on the stigma (Morse, 1987; Niesenbaum, 1992), those that can germinate in vitro (Beardsell *et al.*, 1993; Lindgren *et al.*, 1995), as well as those that can absorb specific stains (Becker and Ewart, 1990; Nyman, 1992), and to indicate successful seed set following the process of pollination (Smith-Huerta and Vasek, 1984).

Research into the variability of pollen grains plays a crucial role in elucidating the mating systems of plants. The diversity of these mating systems significantly affects the variability and differentiation processes observed within populations of both wild and cultivated species (Grant, 1981; Richards, 1997). Furthermore, pollen viability is a key factor in addressing issues related to sterility, hybridization initiatives (Stone *et al.*, 1995), and the field of evolutionary ecology (Thomson *et al.*, 1994). This viability is particularly vital for understanding genetic variability in plants where cross-fertilization is more prevalent than self-fertilization (Divakara *et al.*, 2010). Generally, a direct correlation exists between pollen viability and the germination potential of pollen across numerous plant species (Stanley and Linskens, 1974).Numerous methodologies have been established to assess pollen viability, utilizing either the dimensions of pollen grains (Kelly *et al.*, 2002) or the dielectric characteristics of cellular membranes (Heidmann *et al.*, 2016; Heidmann and Di-Berardino, 2017). Nevertheless, even with the advent of innovative techniques, a significant portion of the scientific community continues to depend on traditional methods that necessitate only basic staining processes and a microscope for manual enumeration (Shivanna and Tandon, 2014).

Staining methods serve as effective tools for assessing the physiological condition of mature pollen grains. The appropriateness of a pollen viability test is contingent upon the specific species, as variations in optimal staining techniques have been documented (Rodriguez-Riano and Dafni, 2000). A range of staining methods has been utilized to evaluate the viability of pollen grains. Among these, acetocarmine, Alexander's stain, and aniline blue are the most commonly employed techniques for estimating pollen viability (Alexander, 1969; Migdałek *et al.*, 2014; Radovid *et al.*, 2017). The underlying principle of these staining reactions is based on the acidic nature of certain plant cell components, which exhibit a preference for basic dyes.The acetocarmine technique is extensively employed to assess pollen viability in both cultivated and wild plant species (Amkul *et al.*, 2016; Rathod *et al.*, 2018). Acetocarmine functions as a basic dye, with iron serving as a mordant. The nucleic acids, which possess a strong negative charge due to the phosphate groups present in the DNA structure, readily interact with basic dyes. Consequently, this method is utilized for swift evaluations of nuclear conditions and for in-depth in-

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vestigations of chromosomes (Ali, 2008).

In Libya, Thyme (*Thymus capitatus*) is native to Al-Jabal Al-Akhdar area where it has been used for flavoring and medicinal purpose. Thyme is a candidate for large scale as a source of nectar for honey bees, and as an aromatic plant containing essential oils, which are important to the flavoring and medicinal applications (Ali and Mustafa, 2019).

Furthermore, an understanding of reproductive biology, particularly regarding pollen viability, is crucial for the conservation, management, and restoration of endangered species (Kuniyal *et al*., 2003; Murugan *et al*., 2006). Given that thyme (*Thymus capitatus*) holds significant medicinal and ecological value, it has garnered substantial research interest (Ali and Mustafa, 2019; Ali and Mustafa, 2021; Ali *et al*., 2022). However, studies on the floral biology of *T. capitatus* have not been performed. Therefore, the objective of this research is to estimate the pollen viability of *T. capitatus* accessions (populations) that are growing in the south El-Jabel El-Akhdar area, Libya.

MATERIALS AND METHODS

Pollen samples of *Thymus capitatus* were collected from southern parts of El-Jabal El-Akhdar area, aiming to cover as much of the geographic range as possible. *Thymus capitatus* that growing in southern parts of Al-Jabal Al-khdar shows flower color polymorphism. There are five different accessions (phenotypes); white- flowered, dotted white-flowered, purple-flowered, violet-flowered and flowered, and mosaic-flowered accessions (Ali and Mustafa, 2019; Ali and Mustafa, 2021).
Thymus capitatus began to flower on in mid-july and generated pollen for approximately four weeks. Branches containing several inflorescences were harvested from 25 plants per accession (genotype) at three distinct periods during the pollination period: 1 week, 2 weeks, and 3 weeks. They were collected between 8:30 and 10:30 a.m. on sunny day mornings. The branches with inflorescences were placed in jars filled with tap water and maintained at room temperature (25 °C ±5 °C).
Pollen viability was determined by staining with acetocarmine (Ali and Bataw, 2014). Acetocarmine stains the pollen grain nuclei, and weakly stains the cytoplasm, and gives a good contrast between the grain and surrounding medium. The pollen nucleus is rich in chromatin material and live pollen stains pink to deep red with acetocarmine, whereas sterile (nonviable) pollen does not take any stain and thus remains almost white and transparent (McKellar and Quesenberry, 1992; Marutani, *et al.*, 1993). To prepare the acetocarmine stain, weigh1gram of carmine powder and dissolving it in 95 milliliters of glacial acetic acid. Subsequently, add distilled water to achieve a final volume of 100 milliliters. The solution should then be boiled, allowed to cool, filtered, and stored in a refrigerator.For the analysis of pollen viability, two to three drops of stain were applied to a slide, onto which pollen grains were subsequently sprinkled. A cover slip was then placed over the preparation, and the viability of the pollen was assessed after a duration of 5 to 10 minutes. For each of the five accessions (genotypes), at least 50 pollen grains were randomly counted in each visual field on the slide using a light microscope at a magnification of 100X. Each accession is analyzed using ten slides, from which images are obtained. That are red are identified as viable pollen. The average quantity of pollen for each accession is calculated, and the viability of the pollen is expressed as a percentage. The percentage of pollen viability is determined using the following formula:

$$\text{Pollen Viability (\%)} = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$$

Utilizing a completely randomized design (CRD), the experiment was set up. Analysis of variance

(ANOVA) was performed on the experimental data. The significant F test (P 0.05) was used to determine the least significant differences.

RESULTS

According to the color of flower, there are five accessions that growing in south of Al-Jabal Al-Akhdar region, Cyrenaica, Libya: white-flowered, dotted white-flowered, violet-flowered, purple-flowered and mosaic-flowered accession (Ali and Mustafa, 2019; Ali and Mustafa, 2021; Ali, *et. al*. 2022).
In the current investigation, pollen viability was tested using acetocarmine stain which yielded a sufficiently clear result as shown in Figure 1. Since nonviable pollen didn't absorb the stain, which contrasted sharply with the red color of viable pollen, aceto-carmine stain was a dependable way to determine whether thyme pollen was viable (non-aborted) or nonviable (aborted); more significantly, the stain never colored aborted pollen (Fig. 1).

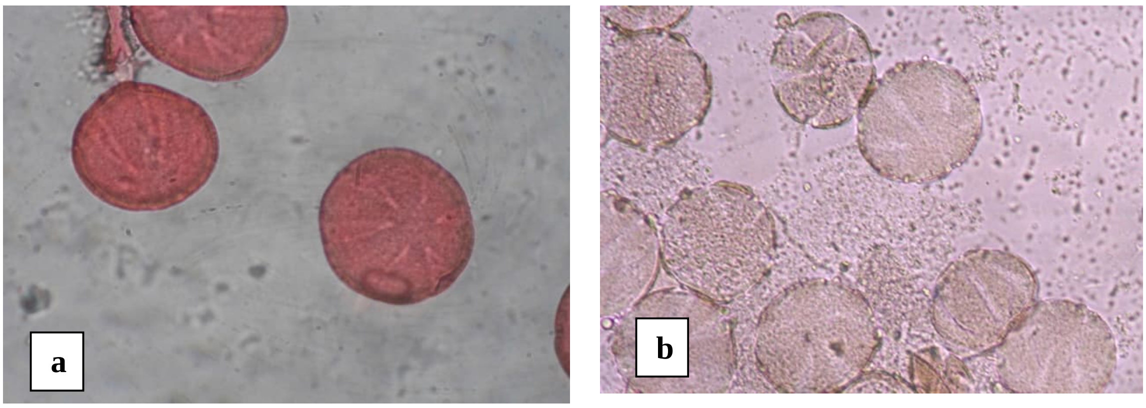

Figure: (1). Pollen grains of Libyan thyme (*Thymus capitatus*) tested by aceto-carmine stain;(a viable pollen, and non-viable pollen b).

Table 1 shows the results of pollen viability of the 5 accessions (genotypes) of Libyan thyme (Thymus capitatus) which growing in southern parts of Al-Jabal Al-Akhdar area, evaluated with aceto-carmine stain. The specimens examined generally produced well-formed pollen which had a high stain ability.The viability of pollen percentage in *T. capitatus* accessions was found to be significantly different (LSD=1.19 at $P \leq 0.05$) in the acetocarmine method. The viable pollen recorded as highest was obtained from the white-flowered population (67.61%) followed by dotted white-flowered population (57.24%), while the lowest viable pollen was obtained from the anthers of mosaic-flowered population (29.86%) in the acetocarmine stain test. The remaining two populations; violet-flowered and purple-flowered showed 55.73% and 42.38% respectively as pollen viability percentage (Table 1 and Fig. 2).

Table:(1). Viability percentages of pollen in libyan thyme (*Thymus capitatus*) accessions

Period of time	Means LSD = 2.08					Means LSD= 0.93
	White-flowered	Dotted white-flowered	Violet-flowered	Purple-flowered	Mosaic-flowered	
1week	78.08^{a}	70.83^{b}	64.45^{c}	52.15^{f}	38.30^{h}	60.74^{A}
2weeks	70.34^{b}	59.34^{d}	61.31^{d}	43.19^{g}	29.91^{j}	52.86^{B}
3weeks	54.48^{e}	41.60^{g}	43.48^{g}	34.89^{i}	21.18^{k}	38.10^{C}
Means LSD=1.19	67.61^{A}	57.24^{B}	55.73^{C}	42.38^{D}	29.86^{E}	

(a-b-c-d-e-f-g-h-i-j-k) These symbols indicate significant differences. If they differ, there is a significant difference. If the symbols are similar, there

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are no significant differences.

Variation was also observed in the pollen viability among different collection periods. The current results indicated that, pollen viability percentage of five accessions (populations) was significantly affected by the collection period (Table 1). The pollen viability percentage began with 60.74% for the first week, then recorded 52.86% for the second week. After three weeks, the pollen viability percentage sharply decreased to 38.10%. Those results were significantly different at the value of L.S.D =0.93, P≤ 0.05.

Furthermore, the three collection periods within each single population (accession) showed different pollen viability (Fig. 2). For each accession, pollen viability was highest in the first collection period (the 1st week) and lowest in the third collection period (the 3rd week). The highest values of pollen viability occurred in the first period of collection (the 1st week) recording 78.08% (white-flowered), 70.83% (dotted white-flowered), 64.45% (violet-flowered), 52.15% (purple-flowered), and 38.30% (mosaic-flowered). In the second period of collection (the 2nd week), the values of pollen viability were 70.34% (white-flowered), 59.34% (dotted white-flowered), 61.31% (violet-flowered), 43.19% (purple-flowered) and 29.91% (mosaic-flowered). The lowest percentages of pollen viability occurred after three weeks (the 3rd period of collection) recording 54.48% (white-flowered), 41.61% (dotted white-flowered), 43.48% (violet-flowered), 34.89.19% (purple-flowered) and 21.18% (mosaic-flowered). Those results were significantly different at the value of L.S.D =0.93, P≤ 0.05.

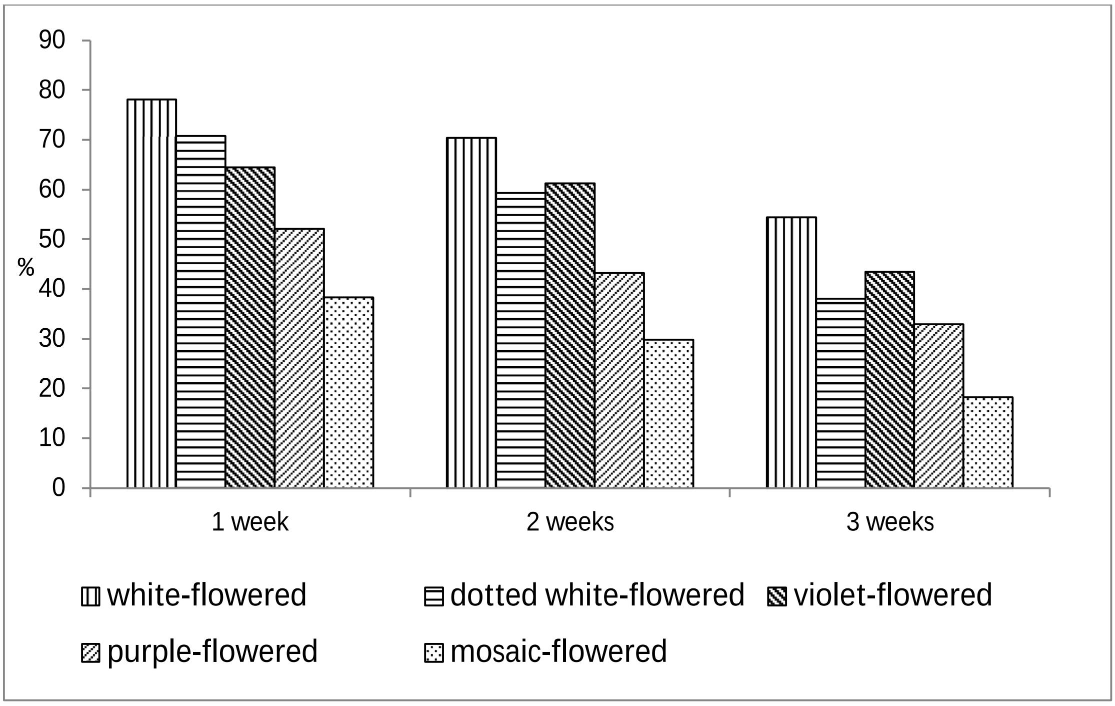

Figure: (2). Viability percentages of pollen during the three collection periods in Libyan thyme accessions

DISCUSSION

The results clearly showed that pollen viability using acetocarmine stain was effective in identifying fertile pollen grains, thus obtaining an early indication of pollen quality. According to several

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previous studies (Thomson, 1989; Dafni and Firmage, 2000; Bots and Mariani, 2005; Ali and Batao, 2014), pollen viability is a crucial indicator of pollen quality. The viability of pollen grains, which can be evaluated using a variety of techniques, including staining with non-vital dyes, is typically a prerequisite for successful seed set (Dafni and Firmage, 2000; Dafni *et al*., 2010; Guoren He *et al*., 2017). One typical method for assessing pollen viability is acetocarmine staining (Migdałek *et al*., 2014; Maryam *et al*., 2015; Vijayakumar *et al*., 2018).

The findings of the current research indicated that the acetocarmine staining technique effectively distinguishes between viable (non-aborted) and non-viable (aborted) pollen grains in populations of Libyan thyme (*T. capitatus*). Also, the results corresponded well with that of Tiwari *et al*. (2014) who evaluated pollen viability in members of *Euphorbiaceae* family using several dyes and concluded that acetocarmine staining method was efficient for distinction of viable and nonviable pollen grains and provided clear results. Thus, acetocarmine staining method can be recommended for testing pollen viability. Results were also in agreement with other studies indicating that the use of different colorants to test pollen viability may give comparable results (Parfitt and Ganeshan, 1989; Ali and Bataw, 2014).

Temperature, moisture, genotypic variations, plant vigor, physiological stage, and flower age all have a significant impact on pollen survival (Adhikari and Campbell, 1998; Ali and Batao, 2014; Shivashankara *et al*., 2019; Parashuram *et al*., 2021). In the current investigation, the rates of pollen viability were significantly differed among all accessions (genotypes) of libyan thyme. The highest viable pollen rate was recorded from the white-flowered population (67.61%), while the lowest was obtained from the mosaic-flowered population (29.86%). Analysis of variance confirmed the significance of these differences, suggesting high variation in pollen viability among accessions with a 95% confidence interval. The different percentage of pollen viability can be attributed to the genotype, since male inflorescences were sampled from the same site and at the same physiological stage. Genotypic differences among accessions in others plant species for pollen viability have been previously reported (Sharafi, 2011; Ali and Bataw, 2014; Lankinen *et al*., 2018).

Pollen viability is a crucial factor that can restrict seed production (Fritz and Lukaszewski, 1989; Dafni and Firmage, 2000). The findings of the current study revealed that in natural populations of Libyan thyme, pollen viability can significantly decrease after a period of three weeks. Consequently, if pollen arrives at the pistil more than three weeks later, successful pollination may not occur. In general, percentages of pollen viability were decreased with time in all accessions of Libyan thyme. Similar results were reported in many previous studies indicating that pollen viability decreased with time (Beyhan and Serdar,2008; Ali and Bataw, 2014; Slomka, *et. al*., 2014; Mantiquilla *et. al*., 2018; Robles-Gonzalea *et. al*. 2019).

CONCLUSION

The main conclusions of the experimental work should be presented. The contribution of the work to the scientific community and its economic implications should be emphasized.

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