

*Full Length Research Paper*

# **Effects of seed coat pigmentation on germination characteristics and antioxidant properties of *Argyrolobium uniflorum* ((Decne.) Jaub. & Spach) in southern Tunisia**

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Received 7 December, 2023; Accepted 28 December, 2023

Seeds of *Argyrolobium uniflorum* (Fabaceae), a spontaneous plant in arid and semi-arid regions of Tunisia, showed a color variability in their seed coats (green, orange, yellow and brown). The influence of the color of seed coat on germination and phytochemical seed composition was evaluated. Germination tests were carried out and the results obtained show that the applied chemical treatment (sulfuric acid) improved germination, independently of the color tested. In addition, this plant germinated at a temperature range of 10 to 30°C, where each color requires a suitable temperature. Brown seeds have the highest percentage of germination. Total polyphenolic contents of different colors of seeds were studied using three solvents. The yellow seeds TPC was found higher than that of the green, brown and orange colors, with the highest values in acetone extract reaching 7.98 and 8.68 mg EAG g<sup>-1</sup> MS. However, the level of antioxidant activity estimated by DPPH (EC<sub>50</sub> = 0.24 to 1.84 mg / ml) also showed that the yellow seeds exhibit the lowest value (EC<sub>50</sub> = 0.24 mg / ml). The highest values of flavonoids were observed in the seed brown with values 3 mg EC g<sup>-1</sup> MS. Germination associate with the colors of coat of seeds were to be a good process to improve the phenolic content and antioxidant activity of *A. uniflorum* seeds.

**Key words:** *Argyrolobium uniflorum*, germination, colors, antioxidant activity.

## **INTRODUCTION**

*Argyrolobium uniflorum* ((Decne.) Jaub. & Spach), a pluriannual herbaceous legume is from North Africa

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(Dekak et al., 2018) and is a pastoral legume that is widely distributed in arid and semi-arid regions in Tunisia (Ferchichi, 1990). It is considered the most promising complement to highly palatable native Werker (Visser et al., 2011). *A. uniflorum* plays an important role in the maintenance of soil fertility, soil coverage and dune stability (Ferchichi, 1990).

In Tunisia, *A. uniflorum* is a species of wide ecological amplitude (Jauffret and Visser, 2003). It is distributed from the semi-arid to the upper Saharan, with a rainfall range of 50 to 600 mm (Chaieb and Boukhris, 1998). Seeds of *A. uniflorum* are represented as a pod up to 3 cm by 3 mm with 6 to 10 seeds. At maturity, the pod opens at two valves each carrying half the seeds (Pottier-Alepatite, 1979; Neffati, 2008). This taxon is cleistogamous and its seed is highly polymorphic (Visser et al., 2011). The seeds are multicolored (yellow, orange, brown and green) very uniform, hard, containing the albumen. The weight of thousand seeds of this species is 1.750 g. It can produce many seeds. The number of one gram can reach 800 to 1000 seeds, with an average of 8 to 9 seeds per pod (Pottier-alepatite, 1979; Neffati, 2008). The propagation seems possible only by sowing (Neffati, 1994).

Successful establishment of plants largely depends on successful germination. However, the presence of hard seed coat is characteristic feature of the most of legume species. Dormancy imposed by a hard seed coat is a part of seed survival strategy in many species (Werker, 1981; Kelly et al., 1992; Bhatt et al., 2016). Several factors such as high soil temperature soil, microbial attack, temperature fluctuations, humid atmospheric conditions and passage through the digestive system of animal scan contribute to increasing coat permeability of legumes and therefore, increase seed coat germination percentage (Baskin et al., 2000; Bhatt et al., 2016).

Polyphenols are a large and diverse group of phytochemicals in plants (Harborne and Williams, 2000). It was reported that polyphenols had strong antioxidant activities associated with their ability to scavenge free radicals, break radical chain reactions and chelate metals (Kaisoon et al., 2011). Today, polyphenols are considered to be the most important dietary antioxidants due to their high antioxidant capacity and presence in plant foods (Kardum et al., 2014). In arid regions of Tunisia many natural antioxidants have already been isolated from different seeds of the plants, *Astragalus gombiformis* (Teyeb et al., 2012) and *Calligonum* species (Dhief et al., 2013). To the researchers' knowledge, there are no study relating the properties of the polyphenols in *A. uniflorum* in arid regions of Tunisia. It could be suspected to study the germination responses of *A. uniflorum* to temperature in relation to the pigmentation affecting their seeds and evaluate their total polyphenolic content and compare the effects of different extraction

solvents on these potentials.

## MATERIALS AND METHODS

### Seed collection and storage

Seeds of *A. uniflorum* used in study were collected in May 2012 from plants growing on Benguerdane (33° 17' 35N) (10° 46' 47 E). After collection, the seeds were stored at 20 and 30°C relative humidity in Seed Bank of Laboratory of Pastoral Ecosystems and Valorization of Spontaneous Plants, Institute of Arid Regions (Médénine, Tunisia), prior to testing and experiments began in December 2016.

### Seed viability test

Tetrazolium test was used to determine seed viability; about 25 seeds with four replicates were taken from each color of seeds according to Tommasi et al. (2006). The isolated embryos (25) were incubated for 1 h in 5 mL of a solution of 1% (w/v) 2, 3, 5 triphenyl tetrazolium chloride (TTC) in phosphate buffer 50 mM, pH 7.3 for 3 to 4 h at 25°C in darkness. Seeds of which the embryo exhibited no overall carmine red staining were scored as non-viable. Finally, the viability percentage (VP) was calculated as the number of viable embryos/total number of embryos × 100.

### Germination under laboratory conditions

The effects of temperature on seed germination were assessed in incubator at 10, 15, 20 25 and 30° (Lumincube II analysis, Belgium, MLR- 350, Sanyo, Japan) in the dark and in sterile Petri dishes of 9 cm diameter fitted with two layers of Whatman N°1 filter paper moistened with 5 ml of distilled water, and four replicates of 25 seeds in each level of treatment. The Petri dishes were observed every two days for further 16 days to monitor the number of germinating seeds. Germination was considered to be the incidence of radical protrusion (> 2 mm) (Kulkarni et al., 2007).

### Seed pretreatment

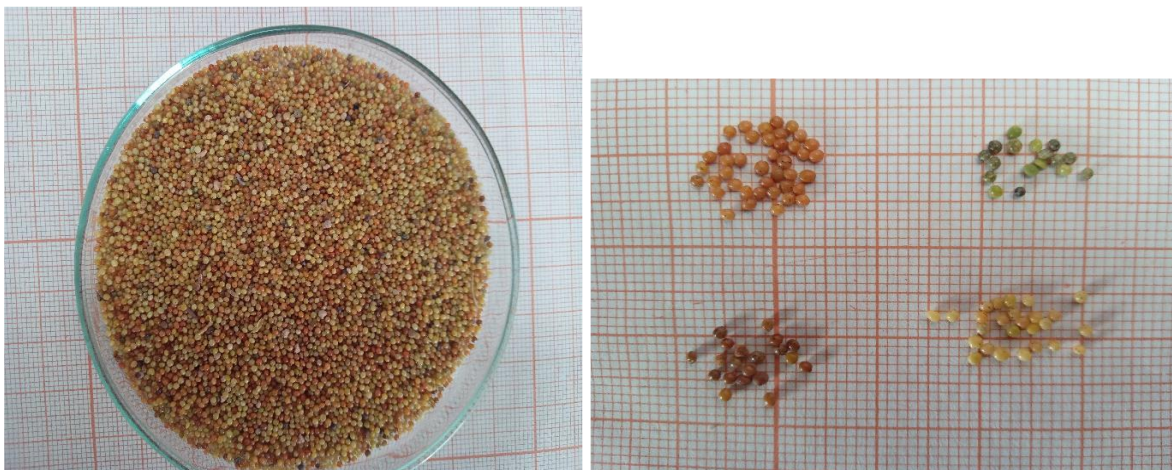
Although seeds varied apparently in color (Figure 1), they received the same sulphuric acid treatment. Seeds were immersed in 70% sulphuric acid for one hour and then rinsed thoroughly in running water for 2 to 3 min and with distilled water, after sterilized seeds had dried.

### Germination characteristics

Three germination characteristics were determined; final germination percentage, cumulative germination percentage which is the cumulative number of germination seeds counted daily and time to reach 50% germination ( $T_{50}$ ). It was estimated according to the formulae of Coolbear et al. (1984) modified by Farooq et al. (2005).

$$T_{50} = t_i + ((N/2 - n_i) * (t_j - t_i)) / (n_j - n_i)$$

where N is the final number of germination and  $n_i$  and  $n_j$  the cumulative number of seeds germinated by adjacent counts at time  $t_i$  and  $t_j$  when  $n_i < N/2 < n_j$ .



**Figure 1.** Phenotype of four representative seeds of each colored *A. uniflorum*. O (orange seeds), Y (yellow seeds), G (green seeds), B (brown seeds).

#### Preparation of seeds extract

Seeds of different colors (yellow, orange, green and brown) are reduced to powder. Polyphenols and flavonoids were extracted using three different solvents; ethanol, acetone and methanol mixed with acetone. A 0.1 g of each sample of different colors of seeds is dissolved in 1 ml of 70% ethanol, 70% acetone or methanol/water (50: 50); v/v). The extracts were centrifuged at 10.000 g for 10 min. At the end, the extracts from ethanol and acetone were recovered. However, the first supernatant from methanol extraction is kept and was added in 1 ml of 70% acetone. A second supernatant is obtained following another centrifugation for 1 h. The two supernatants are stored at -20°C overnight for use.

#### Extraction of polyphenolic compounds

The polyphenol content of the different colors of seeds was determined by the method of Singleton and Rossi (1965) using Folin- Ciocalteau. A volume of 150 µmol of each extract was mixed with 2400 µmol of distilled water, 150 µmol of Folin- Ciocalteau reagent and 300 µmol of 2% Na<sub>2</sub>CO<sub>3</sub>. The solution was adjusted to a final volume of 3 mL, with distilled water and mixed vigorously. After incubation at room temperature 50°C for 2 h, the absorbance was read at 725 nm using a Milton Roy 601 UV- vis spectrophotometer. A calibration curve with gallic acid at different concentration of 0, 0.01, 0.025 and 0.1 mg/ml. The polyphenols contents are reported in mg gallic acid equivalents per g of dry matter (mg EAG/g DM).

#### Total flavonoid content

The total flavonoid was extracted by spectrophotometrically with aluminum chloride using the method of Zhuang (1992). The method was based on the formation of a complex flavonoid aluminium having maximum absorbance at 415 nm. Firstly, 300 µL of each sample were adjusted with 600 µL of distilled water, 45 µL de NaNO<sub>2</sub> (5%) and 45 µmol of 10% methanolic aluminium chloride ALCL<sub>3</sub> solution. After 2 min of a stand at room temperature, the absorbance was measured at 415 nm using spectrophotometer.

Total flavonoid contents were calculated as quercetin equivalents (RE) from a calibration 0.005, 0.01, 0.05, 0.1 and 0.2 mg/ml and expressed as mg RE/100 g DW. All measurements were performed in triplicate.

#### Diphenyl radical scavenging activity assay (DPPH)

DPPH (2,2-diphenyl-1-picrylhydrazil hydrate) radical scavenging activity was determined according to the method of Amarowicz et al. (2004) that is slightly modified by Yesil-Celiktas et al. (2007). A lot of 100 mg of seed FW was homogenized with 2 mL of methanol and centrifuged at 9,000 g for 15 min. To 100 µL of extract, 2.9 mL of 0.1 mM methanolic solution of DPPH was added. The contents were stirred vigorously and then left to stand at room temperature for 30 min in dark. Decrease in colorization was measured spectrophotometrically at 517 nm. The radical scavenging activity (RSA) was calculated using the equation:

$$\text{RSA (\%)} = 100 \times (1 - \text{AE/AD})$$

where, AE represent the absorbance of the solution containing antioxidant extract and AD is the absorbance of the DPPH solution. All measurements were done in triplicates.

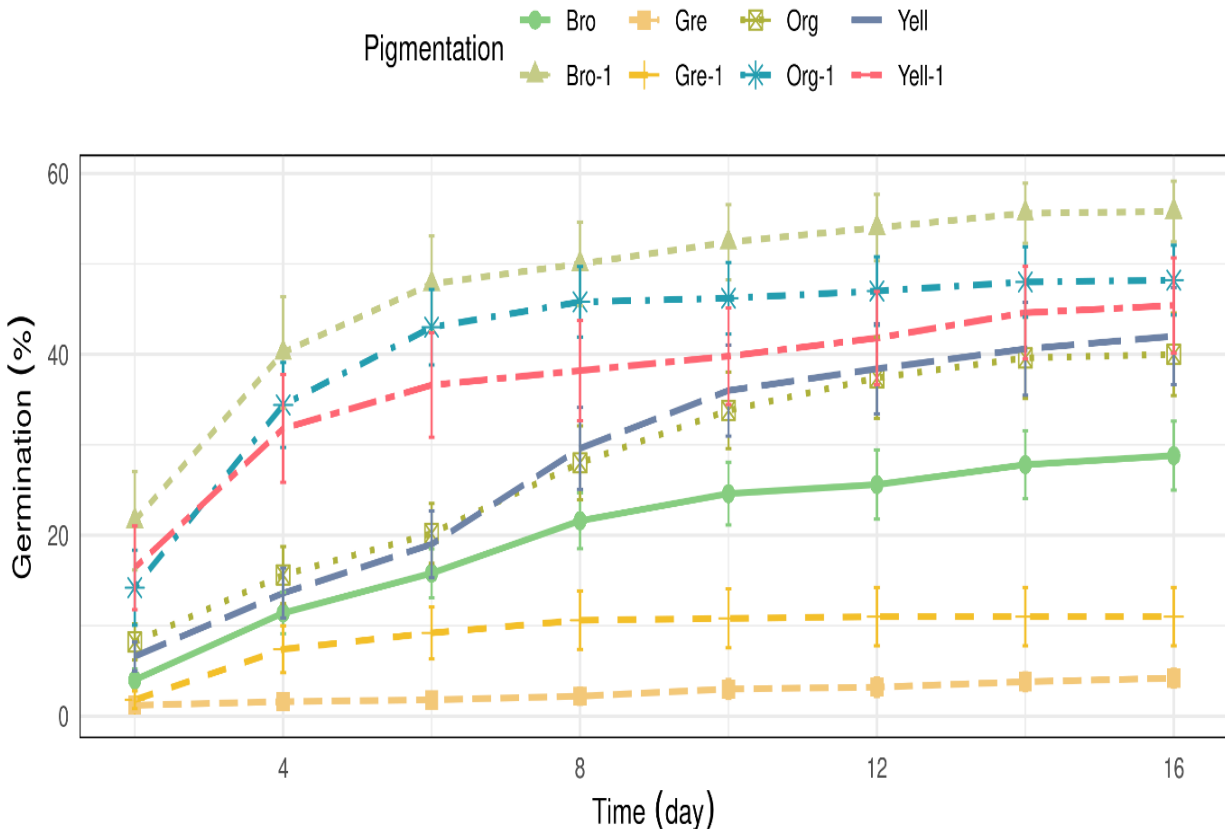
## RESULTS

#### Seed viability test

The viability test shows that the brown seeds of *A. uniflorum* recorded the highest percentage of viability (98%) compared to those of yellow color seeds and orange color seeds (97%). Indeed, the seeds of green color presented the low percentage of viability (96%).

#### Seed germination

The germination responses of the different colors of *A.*



**Figure 2.** Cumulative germination percentage of withness and treaty of four colors ((A) orange, (B) yellow, (C) green, (D) brown) of *A. uniflorum* seeds for 16 days at different temperatures (10, 15, 20, 25 and 30°C) (n=4).

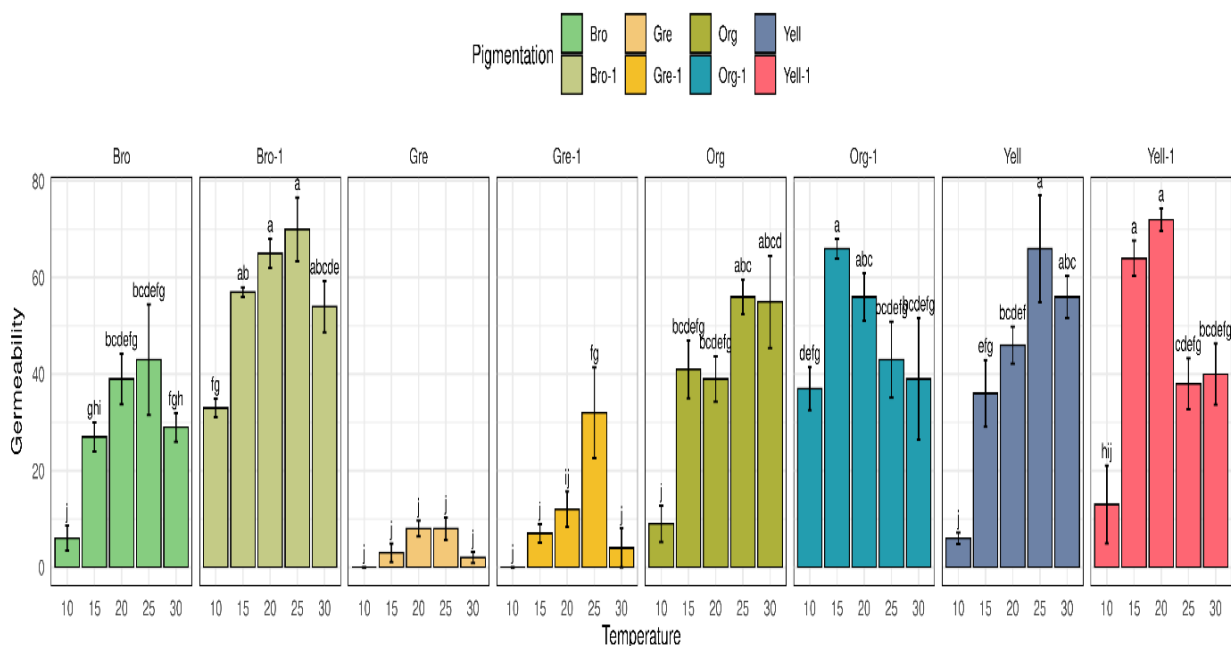
*uniflorum* (control and treated) to a wide range of temperatures (10 to 30°C) are illustrated in Figure 2. At these temperatures, the final germination percentage of different colors of seeds (control and treated) were higher than 50%. Incubation temperature from 10° to 30°C was suitable for germination of *A. uniflorum*. The germination percentage of the control seeds of the different colors presented more or less variable rates. This percentage varying between 0 and 56%, is recorded in green and orange seeds respectively. The delay of germination (DG) decreased with increasing temperature; it varied between 2 and 4 days in all control seeds. The study recorded only 2 days for temperatures 20, 25 and 15°C and it was delayed for 4 days at 10°C.

The final germination percentage of treated seeds of different colors ranging between 4 and 72%, recorded respectively in treated green seeds and yellow seeds. The delay of germination (DG) varied between 2 and 6 days respectively in treated orange seeds and brown seeds and between 0 to 8 days respectively in yellow and green seeds. Figure 3 illustrated that the germination percentage of treated seeds of *A. uniflorum* of all colors

remains between 15 and 20°C with a percentage that did not exceed 70%. At 10°C the germination percentage is low; 0% was recorded in green seeds color at all temperatures tested (10 to 30°C) the treated brown seeds exhibited high germination percentage recorded at 25°C.

The curves relating to the mean MTG germination time (Figure 5) show that the germination rate of the treated and control seeds decreases as one moves away from the temperatures allowing the highest germination rates to be obtained and that this reduction in speed is generally greater for the lower temperatures than for the higher temperatures.

Temperature was significantly affected the percentage of germination of the treated and control seeds of all the colors ( $P < 0.001$ ) and showed a non-significant effect at the 5% on delay of germination for all the seeds colors (Table 1). Two-way ANOVA (Table 2) indicated a highly significant effect shows that in all the colors of the seeds tested the effect of the treatment on the percentage of germination of the different colors of seeds ( $P_{0.003}$ ) and on delay of germination.



**Figure 3.** Seeds germination of different colors of *A. uniflorum* withness and treaty; O (orange seeds), Y (yellow seeds), G (green seeds), B (brown seeds) at different temperatures of incubation (10, 20, 25 and 30°C).

### Extraction of polyphenolic compounds

The TPC of *A. uniflorum* seeds of different colors (brown, yellow, green and orange) using the three solvents as shown in Figure 4. The results indicated that the highest amount of total phenolic compounds (7.98 and 8.68 mg EAG g<sup>-1</sup> MS) is obtained for the acetone extracts by comparison with the other extracts (ethanol or mixture of methanol: acetone) (Figure 5). In the different seed colors, the polyphenol contents obtained from the ethanoic extracts vary between 6.92 and 7.42 mg EAG g<sup>-1</sup> MS. Nevertheless, these values remain low (6.79 and 7.49 mg EAG g<sup>-1</sup> MS) from the methanol: acetone extracts and those in all the different colors of seeds. It thus appears that the yellow color of seeds is the richest in polyphenols.

### Total flavonoid content

Analysis of the results in Figure 4 shows that the green seeds have the highest content of flavonoids compared to the other colors tested. The lowest content was registered in the orange seeds. This figure shows the importance of the ethanoic extract fraction registered in the seed yellow and seed orange shows the extracts similar values in the order of 2.5 mg EC g<sup>-1</sup> MS, while the seed brown color reaches slightly higher values (3 mg EC

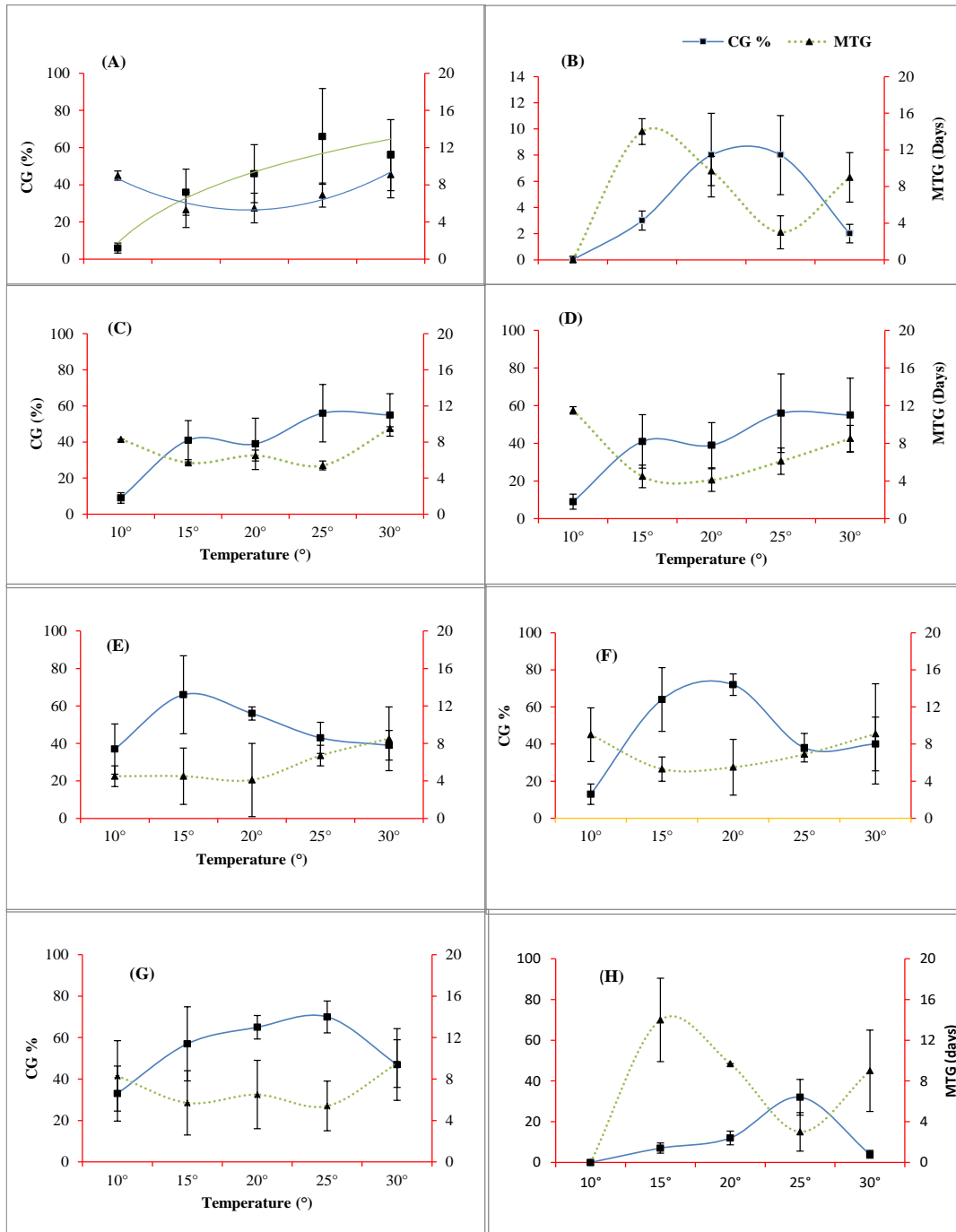
g<sup>-1</sup> MS). In the case of acetone extracts, the flavonoid contents are similar for orange, yellow and brown seeds (3 and 4 mg EC g<sup>-1</sup> DM). Furthermore, the contents of the methanoic extracts are all less than 2 mg EC g<sup>-1</sup> DM. As a result, acetone followed by ethanol are the best solvents for extracting flavonoids compared to methanol.

### Diphenyl radical scavenging activity assay (DPPH)

The antioxidant activities of different colors of seeds of *A. uniflorum* extracts were assessed and confirmed using one functional analytical method based on the anti-radical activity DPPH (Figure 6). The results illustrated in this figure show that the fraction of the acetone extract exhibits the higher activity than other extracts with EC<sub>50</sub> values varying from 0.24 to 1.84 mg/ml (Figure 7). It should be noted that the acetone extracts from the yellow seeds have the lowest EC<sub>50</sub> concentration (0.24 mg / ml). The solvent effect allowing to order the extracts as follows: acetone > ethanol > methanol.

### DISCUSSION

These studies investigated that all the colors of seed coat of *A. uniflorum* were able to germinate over in range of temperatures varied from 15 to 30°C. Similar germination



**Figure 4.** Variation of the cumulative germination percentage (CG) of four colors (A) yellow, (B) green, (C) brown, (D) orange) of withness and ((E) yellow, (F) green, (G) brown, (H) orange) of treated seeds of *A. uniflorum* and the speed of germination (MTG, days) according to the different temperatures (10, 15, 20, 25 and 30°C).

capability was observed in different southern Tunisia species (*Phragmites australis*, *Diploptaxis harra*, *Lotus creticus* and *Ziziphus lotus*) (Tlig et al, 2008; Gorai et al.,

2009; Rejili et al., 2010; Zammouri and Neffati, 2021). The optimum germination in brown and green colored seeds are recorded at the temperature 25°C, it was 30°C

**Table 1.** Analysis of variance (two way-ANOVA) of the effects of temperature on germination percentage and delay of germination of different colors (green, yellow, brown and orange) of seeds (control and treated) of *A. uniflorum*.

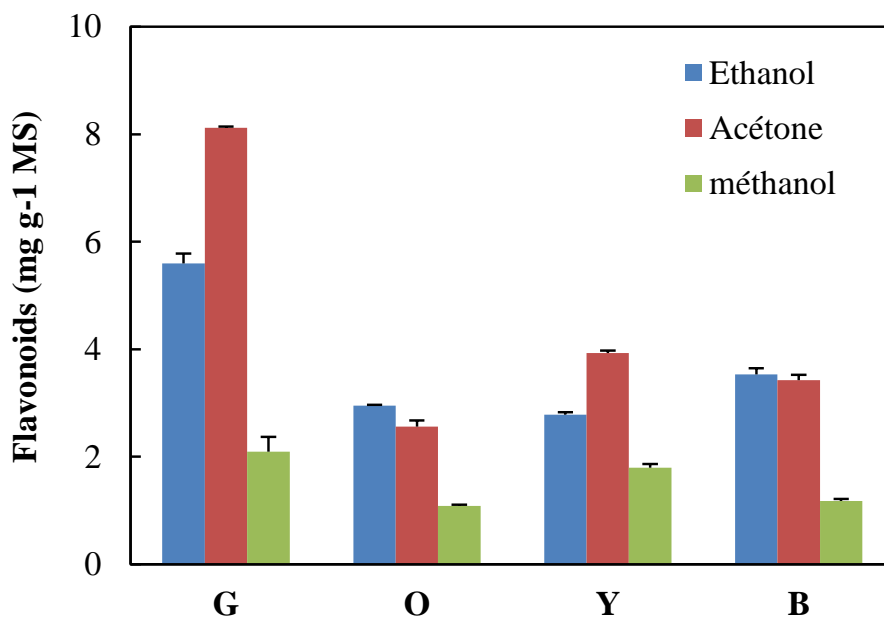
Variable	df	MS	P level
GP	3	801,440	0.000***
DG	3	3,492	0.784 <sup>NS</sup>

GP: Germination percentage; DG: Delay of germination. \*\*\* P<0.001; NS: Not significant.

**Table 2.** Analysis of variance (two way-ANOVA) of the effect of treatment (control or treated) on germination percentage and delay of germination of different colors of seeds (green, yellow, brown and orange) of *A. uniflorum*.

Variable	df	MS	P level
GP	1	327,756	0.003***
DG	1	65,025	0.009***

GP: Germination percentage; DG: Delay of germination. \*\*\* P<0.001

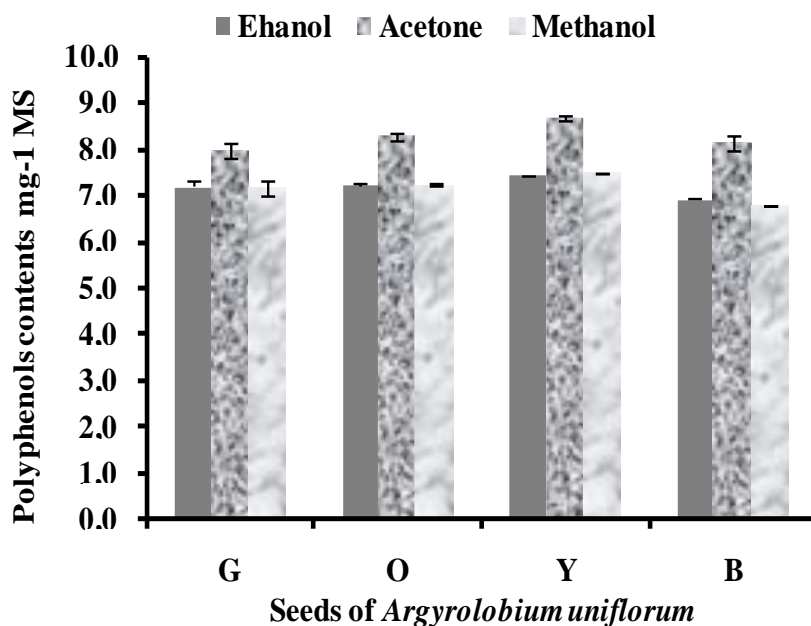


**Figure 5.** Total flavonoid extracts in the different colors of seeds of *A. uniflorum*.

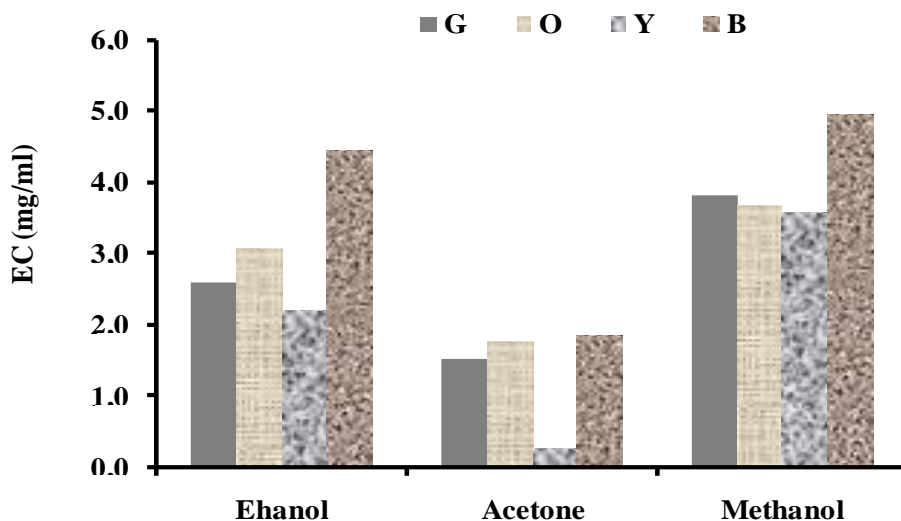
for the yellow seeds and we recorded a thermal optimum 20°C for the orange colored seeds (Figure 4). Further, the study demonstrated that the recorded germination higher capacity did not exceed 70% for all the colors of the seeds treated. Similarly, variable germination capacities were recorded between the treated seeds and

the control. Nevertheless, this germination percentage did not exceed 56% for the control.

It is noted that the treated seeds of brown color have a significantly higher germination percentage compared to the other seeds (yellow, orange and green). Nevertheless, the allowed value of germination in control seeds (not



**Figure 6.** Total polyphenolic content (TPC) of different colors of seeds of *A. uniflorum* expressed as mg catechol equivalent/100 g dry material. For extraction, three different solvents were used.



**Figure 7.** Antioxidant activity of the different colors of seeds of *A. uniflorum*: G (green seeds), O (orange seeds), Y (yellow seeds) and B (brown seeds).

treated) is considered a typical behavior of Mediterranean plants, with optimal germination occurring at temperatures ranging between 15 and 20°C (Baskin and Baskin, 1998). In contrast, low germination percentage of seeds associated with physiological dormancy was observed in *A. uniflorum* as well as in different species

Fabaceae (Gama-Arachchige et al., 2013), this dormancy has been described by Gómez-Maqueo et al. (2021) in many species of this family. The results obtained demonstrate the capital role of sulfuric acid in removing the inhibition of the seed coat. In fact, immersing the seeds for 1 h in 70% sulfuric acid makes it possible to



obtain the highest germination rate and a reduction in the average germination time.

Under controlled conditions (laboratory) germination of the different colors of seeds of *A. uniflorum* are higher at temperatures ranging from 15 to 30°C and that the low germination capacities are recorded at 10°C. This result shows the importance of dormancy for the survival of this species, which maintained high viability even after the storage period (5 years). Moreover, these seeds are orthodox, which persist for long periods in *ex situ* seed banks, it can be quiescent or dormant, and they have very scarce metabolic activity that is activated to germinate in the appropriate environmental conditions, mainly determined by the availability of the water and by the temperature (Gómez-Maqueo et al., 2021).

Previous studies on the activity antioxidant properties obtained from *A. uniflorum* revealed that it displays antioxidant activity. These results are in accordance with the findings of other authors concerning phenolic contents and antioxidant activity in valuable species in arid Tunisian regions such *Lotus creticus* L. (Mahmoudi et al., 2020), *Periploca angustifolia* Labill. (Abdellaoui et al., 2013), *Polygonum equisetifolium* (Mahmoudi et al., 2019), and *Allium roseum* L. (Najjaa et al., 2011).

The data analysis reveals that the yellow seeds of *A. uniflorum* presented a high rate of polyphenols which have been correlated with their high percentage of germinated seeds (38 to 72%) and a phenolic content ( $R_2=0.868$ ). Many researchers showed the role of phenolic compounds in maintaining the viability of seeds. The results are similar to those reported by Abdellaoui et al. (2013) on *Periploca laevigata* L. seeds stored from one to 15 years showing that germination capacity was strongly and positively correlated with amounts of total phenolic compounds. Bailly (2004) has suggested that the dormancy can be alleviated with oxidants, which can oxidize the phenolic compounds present in the seed envelopes, and may allow improved oxygenation of the embryo during seed imbibition. It can also cause cracking in the coat of hard seeds, thus facilitating their imbibition (Chien and Lin, 1994).

Reports on flavonoids possessing various biological activities such as anti-inflammatory, wound healing, antiulcer, hepatoprotective, anticancer, neuroprotective, antibacterial, antidiabetic and antithrombotic are available in the literature. Oxidative stress and antioxidant defense mechanism may also be a contributing as they are linked with inflammatory conditions (Pietta, 2000; Nayeem et al., 2022).

In this study, the reduction activity of the DPPH free radicals in the ethanolic extract of *A. uniflorum* seeds, estimated by the concentration of inhibition ( $IC_{50}$ ) varies between 2.5 and 4 µg/ml. These ranges of variation were higher than those reported to *A. gombiformis* seeds ( $76.41 \pm 3.72$  µg/ml) and (from 4.71 mg QE/g DR to

72.79 mg QE/g DR) in seeds of *Capparis spinosa* (Lekmine et al., 2020; Tlili et al., 2015). Moreover, the DPPH scavenging activity of *A. uniflorum* seeds showed a small change with the color of coats of seeds.

Flavonoids are among the most important phenolic compounds that may exert several biological activities including antioxidant, antiviral, anti-allergic, anti-inflammatory, antimicrobial, antitumor, hepatoprotective and vasculoprotective (Bruneton, 1999; Seyoum et al., 2006). These properties were largely attributed to the antioxidant potential of flavonoids, ensuring free-radical scavenging activity and protection against oxidative stress (Xu et al., 2007). The remarkably high level of flavonoids ensures *A. uniflorum* seeds as a considerable therapeutic value promoting their use in nutritional and pharmaceutical industries.

Therefore, antioxidant compounds and enzymes have been widely considered as being of particular importance for the completion of germination. The antioxidant compounds  $\alpha$ -tocopherol (Simontacchi et al., 1993, 2003; Yang et al., 2001), flavonoids and phenolics (Simontacchi et al., 1993; Andarwulan et al., 1999; Yang et al., 2001) increase during germination. In this study, the DPPH free radicals in the acetone extract of *A. uniflorum* seeds, estimated by the concentration of inhibition ( $EC_{50}$ ) varies between varying from 0.24 to 1.84 mg/ml. These ranges of variation were higher than those reported to *Calicotome villosa* (16.5 and 22.0 µg/ml). These ranges of variation were higher than those reported to *Retama raetam* (25.16 µg/ml) and *Prosopis farcta* (455 µg/ml) (Boughalleb et al., 2019; Tlili et al., 2015). Moreover, the DPPH scavenging activity of *A. uniflorum* seeds showed a small change with the colors of coats of seeds. The results showed that *A. uniflorum* seeds, due to their phenolic content and high antioxidant activity, could be a prospective source of natural bioactive molecules that could replace synthetic antioxidants, but at the same time, they may point to a source of easily accessible natural antioxidants that could be used as a forage with an important palatability factor influencing yield quantity and quality.

## ACKNOWLEDGMENT

This study was supported by the ERANETMED2-72-303 PACTORES project financed by the Ministry of Higher Education and Scientific Research of Tunisia.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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