# Hypericin from *Hypericum triquetrifolium* in wild and under cultivation: variation revealed by genetic distance

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## Abstract

Hypericin content and genetic diversity were studied in 27 wild populations of *Hypericum triquetrifolium* in Jordan. The wild populations were explored from arid to semi-humid areas (176 to 582 mm), growing in varied altitude (341 to 1577 masl). Hypericin content significantly varied among wild populations (0.03 to 0.14%), and negatively correlated with rainfall indicating increased percentages of hypericin in arid environments. Five populations with high hypericin contents were introduced for cultivation in 2006 and 2007. They were behaved differently and categories into (1) no increase in hypericin content over the wild, (2) continuous increase and (3) decrease then increase above the wild hypericin content. Genetic diversity among the wild populations was high, which distinguished populations into 5 clusters according to their geographical origins. A cultivated two years old Ramtha population significantly contained the highest hypericin content (0.156%). Ramtha superiority alleviated genetically by its clustering pattern and its genetic distance from the other populations. *Hypericum triquetrifolium* wild populations are potentially important source for hypericin that encourage their improvement and cultivation.

*Keywords.* HPLC, hypericin, RAPD, wild population, cultivation.

**Introduction**

*Hypericum triquetrifolium* Turra (Guttiferae family) is a medicinal plant grow wild in Jordan and known locally as Roja, Halawa (= sweet), Aran, and Orayna. Hypericin which is found in *H. triquetrifolium* has been reported to possess antiviral, antibacterial, antipsoriatic, antidepressant and antitumoral activities (De Clercq 2000; Said et al. 2002 and Kizil et al. 2004). Compared to *H. perforatum*, the main and traditional source of hypericin, *H. triquetrifolium* considered as one of the best sources for hypericin from the genus *Hypericum* (Alali et al. 2004).

In USA, hypericin content was studied from wild *H. perforatum* plants collected from different sites in California, Montana and Oregon (Walker et al. 2001). Significant differences were found among the different regions. California samples had higher concentrations of hypericin than those from Montana, whereas Oregon samples did not consistently differentiate from those of Montana and California. Oregon samples mean floral concentration of hypericin was 0.06% and pseudohypericin 0.29%, whereas mean leaf concentrations were 0.04% and 0.19%, respectively. Others, in USA studied *H. perforatum* plant samples of 8 wild populations collected from Montana and California (Sirvent et al. 2002). They recorded concentrations of hypericin ranging from 0.0003% to 0.1250%, while the pseudohypericin ranged from 0.0019% to 0.8458%. Wild *H. triquetrifolium* was studied for hypericin contents in different countries, in Jordan unexpected high percentage (0.43%) was reported by Alali et al. (2004), in Bulgaria 0.04% (Kitanov 2001), whereas in Turkey no hypericin was detected (Crockett et al. 2005).

Limited research has been conducted on the chemical analysis and hypericin content of *H. triquetrifolium* and no previous studies were reported to explore the genetic diversity of this plant. The objectives of this research were to study the hypericin content in wild populations of *H. triquetrifolium*; to investigate the changes in hypericin contents under

cultivation and to study the genetic diversity among the wild populations to uncover genetic relationships between collected populations.

## Materials and Methods

*Collection Sites*

Six areas in Northern, Central and Southern Jordan were selected. These were: Irbid and Ajlun in the North, Salt and Madaba in the Center, and Tafilah and Shawbak in the South. Three to six sites were selected within each area to represent different rainfall zones and altitudes which resulted in 27 different sites for *H. triquetrifolium*. The global position system (GPS, GARMINOlathe, USA) was used to record the sites' coordinates. Rainfall long-term averages were obtained from Jordan Metrological Department.

*Hypericin Analysis*

In July at flowering stage, 10 random *H. triquetrifolium* plants were selected from each site. Plants were dried at room temperature and the top 2/3 aerial parts (harvestable parts) which contain leaf, stem and flower were collectively grounded with a laboratory mill (Thomas-Wiley-Scientific-USA) into 1 mm. Analysis protocol for *H. triquetrifolium* which was reported by Alali et al. (2004) was followed.

*Extraction*

Three random samples from each grounded material were extracted in methanol then quantified for hypericin content using High Performance Liquid Chromatography (HPLC). Inter-population variability was determined statistically according to the completely randomized design (CRD) with three repetitions. About 1000 mg (±0.1 mg) of ground material was weighed and placed into a 100 ml round bottom flask fitted with a reflux condenser. The contents were refluxed for 20 min using 80 ml methanol. Samples were filtered through cotton wool and the filtrates were saved. Plant residues and cotton wool were

Amplification reactions were performed in a thermocycler (PTC-200 MJ Research, USA). The conditions for amplifications were as follows: initial denaturing step at 94°C for 2 min, followed by 35 cycles of denaturing step at 94°C for 40 sec, primer annealing step at 34°C for 40 sec, ramp rate 0.3°C $sec^{-1}$, and elongating step at 72°C for 2 min. The reactions were accomplished by a final elongating step at 72°C for 5 min.

Following amplification, the reaction mixtures were fractionated by electrophoresis (Apelex, France) on 1.5% agarose gels (stained with in ethidium bromide, 2 μg/ml) and in 0.5 × TBE buffer at 5 V $cm^{-1}$. A 100 bp DNA Step Ladder (GenScript Corp, USA) was used as the size marker in all electrophoresis. The gels were photographed under UV light (254 nm) using a gel documentary system (Vibber Lourmat, France).

*Data analysis*

RAPD amplified products were scored as present (1) or absent (0) of DNA bands for each sample by visual inspection of the gel photograph. The presence or absence of an amplified fragment was treated as an independent character without considering the qualitative aspects of the results, i.e. band intensity. Pair-wise comparisons of populations, based on unique and shared polymorphic products, were used to generate Nei genetic distance (Nei 1972) then the genetic distance coefficients were used to construct the dendrogram, using NTSYSpc (version 2.1) numerical system (USA)

*Cultivation*

Five populations of *H. triquetrifolium* (containing the highest hypericin contents) were introduced to cultivation, namely; Ramtha, Airport, Qadessya, Shawbak and Mushager. Seeds were scarified with sand and washed several times in water, then sown in polystyrene trays containing peatmoss in the greenhouse. When seedlings were 5-8 cm tall (early May), they were planted by hand at Mushager Agricultural Research Stations field (belong to NCARE, Center of Jordan). The experimental plot (received single population) was 1.2 m × 5 m,

consisted of four rows 5 m long and 0.30 m apart. Each plot was sub-divided into two sub-plots (2.5 m long). Fertilization treatments of 200 kg N $ha^{-1}$ (urea 46% N) and zero were added. Experimental design was split block arrangements in randomized complete block design (RCBD) with three replicates.

*Statistics*

Statistical analysis and comparisons between means were conducted using least significant differences (LSD) at 0.05 probability level (Steel and Torrie 1980). Correlation between hypericin content and site growing conditions (rainfall and altitude) were conducted.

**Results**

Wild populations of *H. triquetrifolium* are adapted to wide ranges of growing conditions. Plants explored from arid to semi-humid areas (176 to 582 mm) and growing at altitude from 341 m to 1577 masl.

*Hypericin content in wild populations*

Hypericin content varied significantly between the wild populations from 0.034% to 0.14% (Table 1). The drier Southern region populations (average rainfall 266 mm) have 15% more hypericin than the grand mean, while the hypericin content from the Central region (average rainfall 347 mm) and the Northern region (average rainfall 393 mm) were close to the grand mean. Airport road (Central region, arid 176 mm) and Qadessya-I (Southern region, marginal 300 mm) contained significantly 101% and 89% more hypericin than the grand mean content, whereas the lowest hypericin content of 0.034% was found in Zay-II population (Central region, semi humid 599 mm). Other populations with hypericin content greater than 0.01% were the Shawbak (Southern region, marginal 394 mm) and Ramtha-I (Northern region, marginal 214 mm) with 52% and 46% increase over the grand mean, respectively.

Correlation between the hypericin content and site rainfall was negative and significant ($r$ = -0.56, $r^2$ = 0.32). On the other hand, no significant associations were found between hypericin content and site altitude.

Position for Table 1

*Hypericin content of cultivated populations*

The 2 years old cultivated populations produced 39% more hypericin than the one year old (Table 2). Hypericin content in the second season increased over the first season by 47, 54, 17, 45 and 33% for Ramtha, Shawbak, Airport, Qadessya and Mushager populations, respectively. In the first season (2006), no significant effect was found for population or fertilization on hypericin content. However, Airport population contained 12% more hypericin than the mean of the populations followed by Ramtha and Qadessya populations, whereas the lowest content was found in Mushager population. In 2007, significant effect was due to the populations only on hypericin production. Ramtha population contained significantly the highest hypericin percentage among the five populations with 10% more hypericin than the mean of the populations followed by Qadessya and Shawbak, whereas Airport and Mushager contained lower hypericin content than the mean of the populations.

*Population's behavior in hypericin production between wild and cultivation*

The five populations of *H. triquetrifolium* behaved differently for their hypericin content in the wild and when they were cultivated in a three different cases (Fig. 1). In the first case, hypericin content in the wild was higher than the cultivated as the case of Airport population. Airport population contained significantly the highest hypericin content (0.14%) among populations in the wild (Table 1), and remained higher than the two seasons of cultivation (0.115% and 0.134% in 2006 and 2007, respectively). Besides, its content of hypericin remained the highest among the populations in the first season of cultivation (Table 2). The second case indicated vigorous response of a population to cultivation and gradual

increase of hypericin content as it was introduced to cultivation, as the case of Ramtha and Mushager populations. Ramtha wild population contained 0.102% hypericin, which increased gradually as the population introduced to cultivation (0.106% and 0.156% in 2006 and 2007, respectively). Similar trend was found in Mushager population; however, Mushager population remained the lowest in hypericin content among the five populations in the wild and under cultivation. The third case was found in Qadessya and Shawbak populations. Qadessya and Shawbak populations contained more hypericin in the wild (0.132% and 0.106%, respectively) as compared to their hypericin content in the first season of cultivation (0.106% and 0.100%, respectively), but their hypericin contents was substantially increased in the second season of cultivation (0.154% for both populations).

*Genetic diversity*

Heterogeneous plants with different sizes, shapes and phenological stages were noticed in the same wild population of *H. triquetrifolium* (Fig. 2). Plants were flowering or had not started flowering while others had mixtures of flowering and/or mature and immature seeds which indicates the presence of morphological and genetic variability (Fig. 3).

On the molecular base, five RAPD primers were used in the amplification reactions to determine the level of genetic variation among *H. triquetrifolium* wild populations (Fig. 4). Markers generated by these primers were employed to compute genetic distance coefficient ($D_a$) values (Nei 1972). Bands ranging in size from 150 to 2000 bp were considered (Table 3) Total of 58 markers across the wild populations (816 scored bands) were produced. Of these markers, 40 were polymorphic and 18 were monomorphic. The total number of markers produced with each primer ranged from 9 for primer OPW-08 to 14 for primer OPW-02. Depending on the primer type, the percentage of polymorphism ranged from 54.6% in primer OPW-10 to 91.7% in primer OPB-20. The total percentage of polymorphism among the populations was 68.97%.

Based on Nei`s (1972) genetic distance coefficient ($D_a$), a genetic distance matrix was constructed to assess genetic diversity among the populations. Results showed that the genetic distances between the studied populations were distributed as close as 0.05 to those genetically divers as 0.49, where the overall mean of genetic distance was 0.24 implying that 24% of RAPD markers were different between wild *H. triquetrifolium* populations.

The regions comparisons showed that within population diversity was the highest with populations collected from Northern region of Jordan (mean $D_a$ = 0.22) whereas lower diversities found in the Central (mean $D_a$ = 0.13) and the southern populations (mean $D_a$ = 0.14).

Comparing the areas indicating that the highest within populations diversity was recorded for those collected from Ajlun (Northern region) (mean $D_a$ = 0.23), while the lowest diversities (mean $D_a$ = 0.11) were from Madaba (Central region) populations.

Comparing the populations indicating that the highest genetic distance (0.49) was found between Kufor Youba (Northern region) and Wadi Musa-I (Southern region), and the lowest genetic distance (0.05) was found between Ramtha-I and Ramth-II populations (Northern region) and between Airport and Mushager (Central region). Conversely and in spite of geographical distance, low genetic distance (0.06) was recoded between Mrajmeh (Central region) and Shawbak (Southern region) populations.

At the highest level of hierarchy in the dendrogram (Fig. 5), *H. triquetrifolium* wild populations were divided into two main clusters. One cluster separated Kufor Youba population in its own group. The second cluster consisted of the rest of the populations which in turn divided into 2 main sub-clusters, each of which also sub-divided into 2 sub-sub-clusters, resulting in 5 different clusters.

Generally, clustering patterns linked the wild populations to their regions and narrower to their areas indicating in turn their geographical separations. Out of the 5

generated clusters, 3 of them were for the populations collected from the Northern region of Jordan showing the highest genetic diversity within those population in their region and confirming the previous results of having the highest mean of genetic distance ($D_a$ = 0.22) among the populations in the Northern region. The remaining two clusters were for populations collected from the Salt area (Central region) while the last cluster merging the whole Southern region populations with those collected from Madaba area (Central region).

The first clusters included populations collected from the Northern region, those were: Ketem, Arjan, Zagreet, Ramtha-I, Ramtha-II, Ebben, Bushra and Rass Moneef. The second cluster has an overlapping region that included populations collected from the Central and Southern regions, those were: Airport, Mushager, Mrajmeh, Husban and Mlah from the Central region, and Shawbak, Qadessya-I, Qadessya-II, Wadi Musa-I and Wadi Musa-II from the Southern region. The third cluster included *H. triquetrifolium* populations collected from the Northern region, those were: Al-Wahadneh and Wadi Rayaan. The fourth cluster included populations collected from the Central region, those were: Zay-I, Zay-II and Kufor Hood. The fifth cluster included a unique population collected from the Northern region, which was Kufor Youba population.

**Discussion**

Wild populations of *H. triquetrifolium* were found to be diverse genetically and in respect to their hypericin contents. Hypericin content in *H. triquetrifolium* wild populations varied largely from 0.03% to 0.14%. The locally obtained hypericin percentages are higher than those reported for the same species elsewhere (Kitanov 2001 and Crockett et al. 2005). Conversely, those percentages are much lower than the previously reported (0.43%) in Jordan (Alali et al. 2004). In the formal report hypericin content was determined separately from the leaves, flowers, and stems and found to be 0.36%, 0.064%, and 0.0085%, respectively. The

reported unexpected high percentage was due to the summation of hypericin contents without considering the percentage of contribution of each plant part to the total plant dry weight. In this study, percentages of each aerial part (leaf, stem and flower) was determined from five plants collected from Ramtha area (same area of formal study) and were 34%, 61.2%, and 4.8% for leaves, stems and flowers, respectively. When the contribution of hypericin to each plant part according to its percentages was added, the total estimation of hypericin will be 0.13% and not 0.43% as being reported, which is in agreement with the findings of this study.

In the present study, Airport population gave the highest hypericin content (0.14%) among populations. This population was collected from the driest area (176 mm). Moreover, populations collected from the driest region in Southern Jordan showed the highest hypericin percentage average (0.080%) followed by those from the Central (0.067%) then the North (0.061%). This trend has been confirmed by the significant negative correlation found between rainfall and hypericin content, which indicated that the arid and marginal areas should be targeted for future collection of plants with high hypericin contents.

High hypericin content (0.16%) was obtained from the two years old cultivated Ramtha population, which significantly exceeded other populations. Comparing hypericin content of populations in wild and under cultivation showed that the most adapted population to cultivation was Ramtha with continuous increase in hypericin percentage as the plant moved from the wild to domestication. Whereas, Airport population (the highest hypericin producer in wild) produced lower hypericin under cultivation.

Genetically, a high percentage of polymorphism was recorded among the populations (68.97%) indicating their broad genetic base. Similarly, in Germany a high proportion of polymorphic markers (93%) were obtained from six different *Hypericum* species using RAPD marker (Smelcerovic et al. 2006). Martin and Bermejo (2000) showed that the higher the percentage of polymorphic markers, the higher the genetic diversity. Clustering patterns

distinguished populations into 5 clusters mostly according to their geographical origins, which indicated a high level of diversity, and reflected their large genetic base in Jordan that make them adapted to varied habitats. More intensely, 3 of the 5 clusters were for populations collected from Northern region of Jordan, indicating also the intra geographical distinction and the geographical restricted evolution, especially in the lonely clustered Kufor Youba population. Similarly, Narain (2000) showed that the high level of genetic variation found in the natural populations indicating that populations have plenty of scope for evolution to occur. A regional overlapped cluster has grouped some populations from Central and Southern regions together. For example, the genetic distance between Mrajmeh (Center) and Shawbak (South) sites was only 6%. Explanation of this could be due to the fact that both sites were grown with wheat which was disseminated to farmers as certified grains between regions by the government, and as *H. triquetrifolium* accompanied the wheat in the fields, it will contaminate the moved wheat grains between the regions.

**Conclusions**

Wild *H. triquetrifolium* populations in Jordan showed broad variability of hypericin content. Hypericin content is significantly and negatively correlated with rainfall, where high hypericin percentages were found in the dryer sites. Hypericin contents of the promising *H. triquetrifolium* populations are comparable to the *H. perforatum* the only global hypericin source. Behavior of the studied populations in respect to their hypericin content varied as they moved from wild to cultivation. There is a need for exploiting other sites basically in arid zones where hypericin content is expected to be high.

**Acknowledgments**

The authors would like to thanks the National Center for Agricultural Research and Extension (NCARE) and Global Environment Facility (GEF) and the World Bank for financing this research through the "Conservation of Medicinal and Herbal Plants Project".

Thanks is extended to Dr. H. Megdade, Dr. M. Oksh, Dr. A. Abdoulat, Dr. A. Quenah, Mr. M. Graybeh, for their technical help.

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**Table 1.** Sites rainfall, coordinates and hypericin content of wild *Hypericum triquetrifolium* populations collected from different areas in Jordan.

| Region /Area | Site/ Population | Rainfall (mm) | Coordinates | | | Hypericin (%w/w) ± SD‡ |
|---|---|---|---|---|---|---|
| | | | Altitude (masl) | Latitude (N) | Longitude (E) | |
| North/ Irbid | Ketem | 400 | 867 | 32° 23′ 59″ | 035 ° 53′ 24.2″ | 0.043 ± 0.001 |
| | Ramtha I | 214 | 614 | 32° 27′ 58.7″ | 035° 57′ 51″ | 0.069 ± 0.015 |
| | Ramtha II | 214 | 614 | 32° 31′ 36.9″ | 036° 2′ 5.4″ | 0.102 ± 0.038 |
| | Bushra | 456 | 516 | 31° 34′ 41.7″ | 036° 57′ 59.8″ | 0.061 ± 0.015 |
| | Kufor Youba | 350 | 510 | 32° 33′ 3.8″ | 035° 33′ 3.8″ | 0.061 ± 0.013 |
| | Rass Moneef | 582 | 1115 | 32° 22′ 54″ | 035° 48′ 53.8″ | 0.058 ± 0.027 |
| | Ebben | 550 | 1000 | 32° 21′ 13.7″ | 035° 49′ 11.9″ | 0.061 ± 0.009 |
| North/ Ajlun | Zagreet | 400 | 852 | 32° 17′ 25.1″ | 032° 51′ 31.6″ | 0.047 ± 0.017 |
| | Arjan | 450 | 710 | 32° 24′ 10.6″ | 035° 44′ 43.2″ | 0.049 ± 0.006 |
| | Al Wahadneh | 400 | 750 | 32° 19′ 36.9″ | 035° 40′ 46.6″ | 0.059 ± 0.011 |
| | Wadi Rayaan | 301 | 341 | 32° 23′ 56.9″ | 035° 41′ 44.8″ | 0.057 ± 0.011 |
| Center/ Salt | Zay I | 559 | 1087 | 32° 4′ 45.9″ | 035° 42′ 57.4″ | 0.045 ± 0.010 |
| | Zay II | 559 | 881 | 32° 5′ 47.3″ | 035° 42′ 57.4″ | 0.034 ± 0.010 |
| | Kufor Hood | 200 | 636 | 32° 3′ 25.9″ | 035° 44′ 34.7″ | 0.064 ± 0.015 |
| | Airport Road | 176 | 783 | 31° 48′ 16.9″ | 035° 54′ 51.1″ | 0.140 ± 0.001 |
| Center/ Madaba | Mushager | 340 | 792 | 31° 46′ 24.2″ | 035° 48′ 5″ | 0.084 ± 0.015 |
| | Mrajmeh | 340 | 745 | 31° 39′ 43.4″ | 035° 46′ 56.7″ | 0.061 ± 0.004 |
| | Wadi Husban | 300 | 710 | 35° 48′ 8″ | 031° 50′ 14.9″ | 0.085 ± 0.007 |
| | Mlah | 300 | 650 | 31° 35′ 57″ | 035° 47′ 13.2″ | 0.060 ± 0.010 |
| South/ Tafilah | Qadessya I | 300 | 1577 | 30° 38′ 35.1″ | 035° 38′ 33″ | 0.132 ± 0.024 |
| | Qadessya II | 300 | 1409 | 30° 40′ 23.6″ | 035° 38′ 33″ | 0.078 ± 0.006 |
| | Dana Reserve | 350 | 1168 | 30° 41′ 14.1″ | 035° 34′ 22″ | 0.041 ± 0.001 |
| | Rwaam | 238 | 1093 | 30° 48′ 34.6″ | 035° 35′ 26.2″ | 0.057 ± 0.005 |
| | Shawbak | 294 | 1458 | 30° 29′ 36″ | 035° 31′ 35.2″ | 0.106 ± 0.005 |
| South/ Shawbak | Fujaj | 294 | 1270 | 30° 31′ 25.4″ | 035° 36′ 41.8″ | 0.067 ± 0.003 |
| | Wadi Musa I | 177 | 1466 | 30° 20′ 34.2″ | 035° 30′ 41.9″ | 0.081 ± 0.005 |
| | Wadi Musa II | 177 | 1417 | 30° 19′ 39.1″ | 035° 30′ 19″ | 0.079 ± 0.025 |
| | **Grand mean** | | | | | 0.070 |
| | ***C.V. (%)*** | | | | | 15.3 |
| | **LSD 0.05%†** | | | | | 0.005 |

*C.V. = coefficient of variation, †LSD = Least significant difference; NS = not significant, ‡SD = Standard deviation.

**Table 2.** Hypericin content of cultivated *Hypericum triquetrifolium* wild populations at Mushager in 2006 and 2007.

| Population | Hypericin content % w/w | |
|---|---|---|
| | 2006 | 2007 |
| **Ramtha** | 0.106 | 0.156 |
| **Shawbak** | 0.100 | 0.154 |
| **Airport** | 0.115 | 0.134 |
| **Qadessya** | 0.106 | 0.154 |
| **Mushager** | 0.085 | 0.113 |
| **Mean** | 0.1024 | 0.1422 |
| **C.V. (%)*** | 8.35 | 12.83 |
| **LSD 0.05%†** | NS‡ | 0.001331 |

*C.V. = coefficient of variation, †LSD = Least significant difference; ‡NS = not significant.

**Table 3.** Total number of scored bands, markers, monomorphic and polymorphic markers and percentage of polymorphism for each primer used in genetic analysis among wild *Hypericum triquetrifolium* populations.

| Primer | Range of Markers (bp) | Number of | | | | Polymorp -hism % |
|---|---|---|---|---|---|---|
| | | Scored bands | Markers | Monomorphic markers | Polymorphic markers | |
| OPB-20 | 200 – 1950 | 110 | 12 | 1 | 11 | 91.7 |
| OPW-02 | 200 – 2050 | 186 | 14 | 3 | 11 | 78.6 |
| OPW-08 | 250 – 1820 | 144 | 9 | 4 | 5 | 55.6 |
| OPW-10 | 150 – 1840 | 191 | 11 | 5 | 6 | 54.6 |
| OPW-16 | 300 – 2030 | 185 | 12 | 5 | 7 | 58.3 |
| Total | - | 816 | 58 | 18 | 40 | 68.97 |

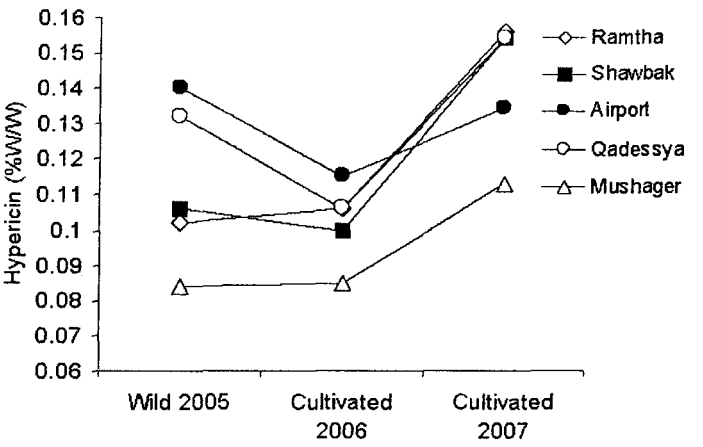


**Fig. 1**. Hypericin content of wild and cultivated *Hypericum triquetrifolium* populations.

**Figure 2**. Varied shape and size of plants (right = general view of the population; and lift = globular plant).

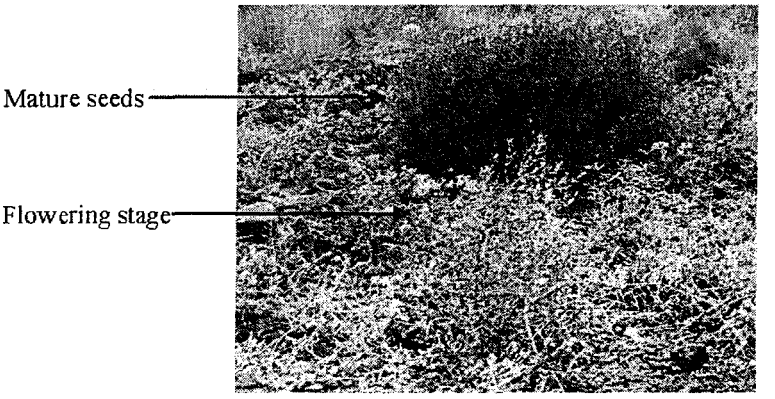


**Figure 3.** Plants with varied phenological stages.

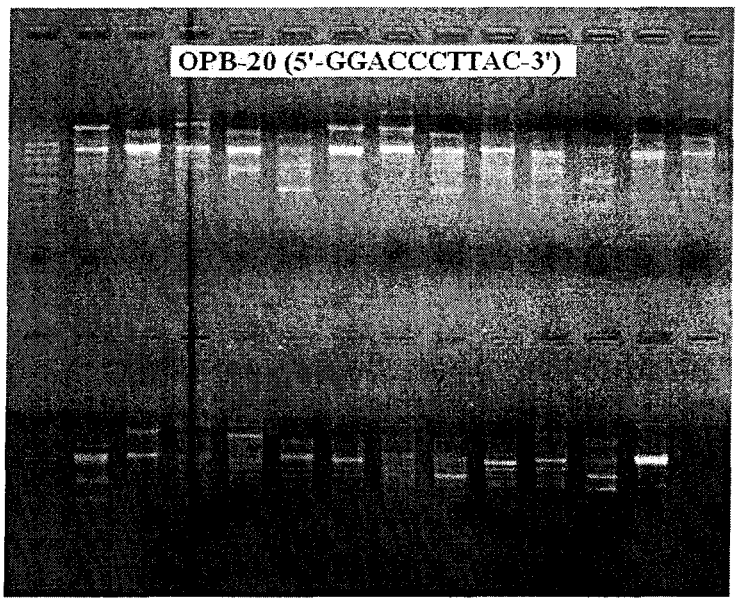


**Figure 4.** Example of amplified DNA patterns using RAPD primers.

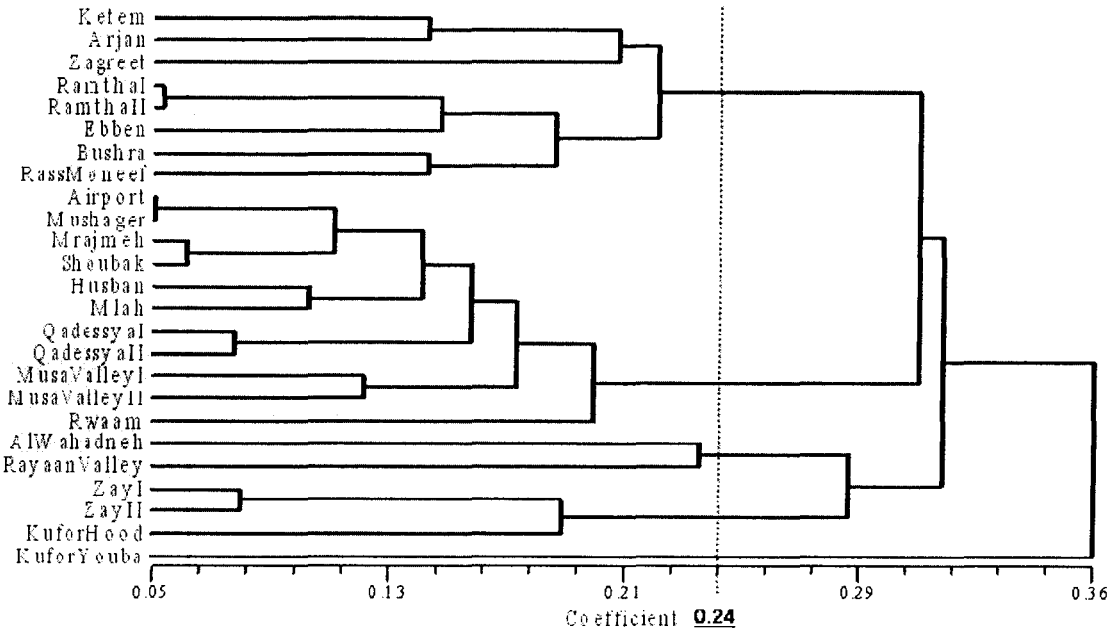


**Figure 5.** Dendrogram of *Hypericum triquetrifolium* populations using five RAPD primers based on Nei genetic distance ($D_a$).