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Essential oil chemical diversity of Moroccan mint (Mentha spicata L.)

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Spearmint (*Mentha spicata* L.) is one of the best known aromatic and medicinal plants. The characterization of essential oil variation is of commercial importance as well as helpful for appropriate use of *M. spicata*. Thirteen strains of cultivated or spontaneous mints were collected from 10 Moroccan regions and acclimatized for two years in Meknes. Their leaves collected at full flowering were dried for hydrodistillation. Essential oil yields were found to vary from 1.32 % to 5.83 %. Analysis of these oils by GC/MS indicated a large variation in chemical composition among the studied strains of Moroccan *M. spicata* (9 to 51 compounds). The highest yields characterize the cultivated strains of Agadir (5.83 %), S_2 of Larache (4.84 %) and the spontaneous strain S_3 of Ifrane (4.17%). The richest strain in number of compounds is S_3 of Ifrane with 51 compounds whereas uncultivated S_2 from the same region has only 9 compounds. Some strains are rich in carvone such as the cultivated strain of Ouarzazate (65.94%) and the spontaneous S_2 of Ifrane (72.3 %). Others are rich in eucalyptol, pulegone or 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) such as those of Marrakech (21.1 %), Agadir (30.7 %) and S_1 of Larache (22.1 %), respectively. The strains of Settat and Ifrane (S_1) have high levels of limonene (17.8-18.1%).

Keywords: *Mentha spicata*, Essential oils, Carvone, Eucalyptol, Cluster analysis, GC/MS

INTRODUCTION

Spearmint, Mentha spicata L cv. viridis (khanuja et al., 2000) or Mentha viridis L. (Chadefaud and Emberger, 1960), is a perennial plant of the labiate or Lamiaceae family (Chadefaud and Emberger, 1960) including 200 genera (Good, 1974; Hedge, 1992; Heywood, 1978). The genus Mentha is composed of 25 to 30 species (Dorman et al., 2003). Some authors present M. spicata as a fertile interspecific hybrid resulting from the crossing of M. suaveolens and M. longifolia (Harley and Brighton, 1977; Gobert et al., 2002). However, some others suggest that there is no genetic evidence for this descent and recommend revising the taxonomic status of M. spicata, including its lectotype (Heylen et al., 2021). Several varieties and hybrids of this species (Gobert et al., 2002; Shasany et al., 2005; Hua et al., 2011; Hua et al., 2012) are cultivated or grow spontaneously on the continents of the globe except Antarctica (Kokkini and Vokou, 1989; Gobert et al., 2002; Liu and Lawrence, 2006; Tucker Arthur and Naczi Robert, 2006). In Morocco, spearmint is cultivated in all regions among which Tiznit, Settat, Meknes and Larache (Tanji, 2008; Eddaya, 2015) characterized by a high production and it also exists in the wild state (Carlier-Loy, 2015). Moroccan spearmint is sold in national markets and also exported to several countries. The production of essential oils is the main objective for the intensification of its cultivation in several countries. Essential oil production of the genus Mentha is ranked second after that of citrus fruits on a global scale (Jullien, 2007).

Mint (M. spicata L.) is one of the best-known aromatic and medicinal plants since antiquity (Tazi, 1986). Indeed, it is used to treat many disorders including the most common ones: colds, coughs, sinusitis, fever, bronchitis, nausea, vomiting, indigestion, intestinal colic, loss of appetite. Obviously, mint is used as a culinary herb to delicately flavour tea, confectionery, chocolates, etc. (Vokou et al., 1993; Lev and Amar, 2000 and 2002; Leporatti and Ivancheva, 2003; Lardos, 2006; Akdoğan et al., 2007; Rhazi et al., 2012; Mahboubi, 2021). These culinary and medicinal properties can be attributed to its richness in several compounds: flavonoids, alkaloids, terpenoids, terpenoid glycosides (spicatoside A and spicatoside B), sterols, steroids, coumarins, caryophyllene, tannins and phenols (eugenol , caffeic acid, rosmarinic acid, α-tocopherol), carvone, linalool, piperitone oxide, carvone-dihydrocarvone, menthone, etc. (Kokkini and Vokou, 1989; Tucker, 1992; Eddaya, 2015). Indeed, many of these compounds are constituents of essential oils used in several fields of economic importance such as medicine, industry, pharmacy and plant protection as biopesticides (Akdoğan et al., 2007; Koul et al., 2008; Omar et al., 2009 Znini et al., 2011; Kapp et al. 2020).

The yield and chemical composition of essential oils of M. spicata are affected by endogenous, and exogenous or environmental factors (Hua et al., 2011; Eddaya, 2015). Indeed, the composition of essential oils depends on the development stage of the plant (Rodrigues et al., 2013; Maffei et al., 1989) and the growth conditions (Maffei and Scannerini, 1999; Karray-Bouraoui et al. , 2009). The flowering period of mint, in summer, is an optimal period for the production of essential oils (Vaverková et al., 2009). The essential oils composition of M. spicata L. accessions from several geographical origins are different from each other (Orav et al., 2004; Oyedeji and Afolayan, 2006; Hajlaoui et al., 2010). This is due to the interaction of the genotype with the various environmental factors (Patel et al., 2015).

In Morocco, few works concerning the essential oils of some cultivated strains have been carried out, to our knowledge, whereas spontaneous strains have never been investigated.

Therefore, this study investigates the chemical variation of cultivated and spontaneous Moroccan mints (M. spicata L.) acclimatized under the same conditions to reveal the role of genotype in their diversity in addition to characterize the chemotypes of the genus Mentha. The present study could contribute to the selection of appropriate strains, thus improving the use of M. spicata L. in pharmaceutical industries and fragrance.

MATERIAL AND METHODS

Plant material

The rooted plants of 13 strains of M. spicata (cultivated and spontaneous) from 10 Moroccan regions (Ouarzazate, Tiznit, Larache, Settat, Agadir, Errachidia, Dakhla, Houara, Marrakech and Ifrane) were planted in March 2016, at the Institute of Horticultural Specialized Technicians of Meknes. The geographical coordinates of the original localities are shown in Table 1. These plants were domesticated for two years before the removal of the leaves intended for essential oils extraction by hydro-distillation.

Three kilograms/strain of fresh leafy stems of acclimatized strains were collected at full flowering stage in July 2018. They were dried in the shade until the weight stability at a relative humidity ranging from 37 to 65% and an ambient temperature below 29°C. The leaves were then separated from the stems and kept cool.

Extraction of essential oils

One hundred grams dry weight (DW) of each strain leaves was subjected to hydrodistillation in 500 mL of distilled water for 3 hours using a “Clevenger” type apparatus. Essential oils collected by decantation at the end of the distillation were dehydrated with anhydrous sodium sulfate and stored in smoked glass bottles in a refrigerator at 4°C. The yield of essential oils is expressed as % (mL/100 g DW).

Analysis of essential oils

Chemical analysis of essential oils was carried out by the technique of gas chromatography coupled with mass spectrometry (GC/MS) at the national center for scientific and technical research (CNRST). The equipment was composed by a chromatograph (Trace GC ULTRA, Milan, Italy) fitted with a VB-5 column (5% phenyl methylpolysiloxane, 30 m x 0.25 mm ID x 0.25 µm) (ValcoBond, Milan, Italy) and an ion trap mass spectrometer. The electron ionization was achieved with an energy of 70 eV (Polaris Q, Milan, Italy). Mass spectra were acquired in a range from m/z 50 to 500 using Xcalibur (version 1.4) software.

One microlitre of a diluted solution (6 mg of essential oils in 1.5 mL ethyl acetate) was injected in split mode with a ratio of 1/50. The carrier gas was helium at a flow rate of 1.4 mL/min. The temperatures of injector and interface zone were respectively 220°C and 300°C. The temperature of ionization source was 200°C. The oven was programmed as follows: The initial temperature was set at 40°C for 2 minutes, then the temperature increased to 180°C at a rate of 4°C/min then to 300°C at a rate of 20°C/ min. The isotherm at 300° C was maintained for two minutes until the end of the test.

Identification of compounds

The identification of essential oils compounds was made basing on mass spectra and their retention indices (RI), according to Adams (2007), which were calculated from the retention time of a series of alkanes (C8-C40). The spectra and the IRs were compared with those obtained from the NIST database (NIST, Version published in 2020) and cited in the literature by several authors (Adams, 2007; Hua et al., 2012; Zhao et al., 2013; Padalia et al., 2013; Soilhi et al., 2019).

Data analysis

The relative percentages of essential oil compounds were treated by various statistical procedures. Cluster analysis was performed using Euclidean distance coefficient functions based on the dissimilarity between pairs. Hierarchical cluster analysis (HCA) was performed using Ward’s method with Euclidean distance measure. The major essential oil components were subjected to principal component analysis (PCA) to reflect the similarities and dissimilarities within the strains. XLSTAT software version 2014 was used for the principal component analysis and the cluster analysis.

RESULTS AND DISCUSSION

Yield of essential oils

The yields of essential oils from the leaves of the different strains studied are very varied. It is clearly higher in the strain of Agadir (5.83%) followed by that of the S2 of Larache (4.84%) and the wild strain S3 of Ifrane (4.17%). On the other hand, it is low in the strain of Ouarzazate (1.32%), the wild strain S1 of Ifrane (1.59%) and the strain S1 of Larache (1.9%). The other strains have a yield that varies from 2.4 to 4% (Figure 1).

This variation could be attributed to the diversity of their genetic heritage, to the environmental factors of regions of origin and/or to their interaction. The study carried out in south-central Morocco revealed the variation in essential oil yield of M. spicata ranging from 1.14 to 3% depending on the sampling region (Eddaya et al., 2015). This variation has been supported by several studies in the case of cultured or spontaneous strains (Telci et al., 2004; Kofidis et al., 2004;

Karousou et al., 2007; Cook et al., 2007; Zhao et al., 2013; Soilhi et al., 2019).

This difference in essential oil content can be attributed to endogenous factors (Dorman et al., 2003; Pompona et al., 2010; Karazmoodeh and Zandi, 2013), to exogenous factors and/or to their interaction (Patel et al., 2015). Indeed, the accumulation and yield of essential oil vary from one hybrid to another, from one strain to another (Farah et al., 2002; Rey et al., 2004; Soilhi et al., 2019), from one variety to another and according to the stage of development (Patra et al., 2001). The essential oil content in M. spicata L. var. Spicata, is high at full flowering (Süleyman et Özlem, 2006). The flowering period of mint, in summer, is an optimal period for the production of essential oils (Vaverková et al., 2009). In addition, several factors inherent to the plant such as leaf or plant age and the genotype affect essential oil accumulation (Kokkini et al., 1989; Kokkini, 1991; Özgüven et al., 1999; Marotti et al., al., 1994; Verma et al., 2010). The variability of essential oil production is accentuated by interspecific hybridization. Patras et al. (2001) found that the interspecific hybrid Neerkalka, resulting from the crossing of Mentha arvensis cv Kalka and Mentha spicata cv Neera, produces annually 271.25 kg/Ha against the production of Neera which is 65 kg/Ha.

The present study showed that M. spicata from Morocco is characterized by a high essential oil yield (1.32–5.83%) for both cultivated and spontaneous strains relatively to spearmint from some countries. Indeed essential oil content of M. spicata accessions from Tunisia ranges from 0.45 to 1.37% (w/w) (Soilhi et al., 2019). That of M. spicata from Mediterranean countries have a wider range from 0.19 to 1.2% (mL /100 g) (Karousou et al., 2007). The essential oil yield of M. spicata accessions from Greece reaches 1.4% (Kokkini et al., 1995) and 1.8% (Kofidis et al., 2004), whereas M. spicata on the island of Zakynthos shows essential oil yields between 0.5 and 1.1% (mL /100 g) (Catherine et al., 2007). Cultivated and spontaneous M. spicata accessions in Turkey show an essential oil content between 1.0 and 2.0%, with a greater variation (1.0–3.0%) in field conditions (Telci et al., 2004). Also for M. spicata from Iran whose essential oil content varies between 0.49 and 1.54 mL/100g (Golparvar and Hadipanah, 2016). However M. spicata accessions from China have a relatively low essential oil yield ranging from 0.5 to 0.8% (Zhao et al., 2013).

Composition of essential oils

The analysis of essential oils of the 13 strains of M. spicata revealed the existence of a high chemical diversity ranging from 9 to 51 compounds identified by strain and which represent 96.9 to 100% of the essential oils (Table 2). These compounds belong to different groups namely hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes, etc.

Similarly to several studies of essential oils composition of Mentha species(Karousou et al., 2007; Mounira et al., 2007; Telci et al., 2010, 2011), oxygenated monoterpenes constitutes the major part with 48,0% to 90,2%, whereas the oxygenated sesquiterpenes vary between 1.7% and 21.2 %. In fact, the oxygenated monoterpenes constitute in wild strain S2 from Ifrane, the largest group with a percentage of 90.2%, whereas monoterpenes and hydrocarbon sesquiterpenes represent only 8.7% and 1.1%, respectively. Other strains are characterized by the presence of a considerable level of sesquiterpenes such as the strains of Marrakech, Tiznit, Settat, Errachidia, Dakhla and Houara.

The majority of strains contain monoterpene ketones and some of them such as that of Ouarzazate and S2 of Ifrane have very high percentages (66.6 to 74.9%) with the dominance of carvone (Table 2). On the other hand, the strain of Tiznit, S1 of Larache and S1 of Ifrane contain monoterpene aldehydes, whereas S1 of Larache, S1 of Ifrane and S3 of Ifrane contain high levels of monoterpene alcohols. The strain of Dakhla is the only one that produces sesquiterpene ketones (Table 2).

Indeed the wild strain S2 of Ifrane is very rich in carvone (72.3 %), followed by that of Ouarzazate (65.9 %) (Figure 2). The strains of Settat, Errachidia, Tiznit, Dakhla, Marrakech and Houara have an average rate of carvone which varies from 22 to 48.5%. The five remaining strains have a very low carvone level ranging from 0.2 to 8.8%. The limonene level is high in the strain of Settat (18.1%) followed by the wild strain S1 of Ifrane (17.8%). The strains of Larache (S1), Marrakech, Dahkla, Tiznit and Errachidia have a limonene level between 10.5 and 13.8%. Other strains have a low limonene rate (3.1 to 9.1%). The strain of Houara does not contain limonene. This variability in essential oils can be explained by interspecific and intraspecific hybridization (Denslow and Poindexter, 2009; Tucker, 2012; Jabeen et al., 2012; Jedrzejczyk and Rewers, 2018).

Some studies have reported that Moroccan spearmint is very rich in carvone with a rate of 62.7 to 75.5% (Eddaya, 2015; Soilhi et al., 2019). It is the major component of the essential oils of M. spicata L. (Govindarajan et al., 2012). Carvone is also mentioned as the main constituent of M. spicata essential oils by many authors (Adam et al., 1998; Mkaddem et al., 2009; Zhao et al., 2013; Brahmi et al., 2016). According to some authors, the level of limonene in Moroccan mint varies between 10.5 and 17.9% (Eddaya, 2015; Soilhi et al., 2019). Noteworthy, strains very rich in carvone are very poor in limonene, such as the wild strain S2 of Ifrane and the cultivated strain of Ouarzazate (Table 2). Indeed in M. spicata, the dominant gene C is responsible for the production of carvone from limonene and the dominant gene Lm inhibits the conversion of limonene to piperitenone with weak conversion of carvone to carveol in the absence of the R gene (Bhat et al., 2002). Hefendehl and Murray, (1972) reported that the essential oils of plants possessing the dominant gene Lm and recessive gene c are rich in limonene, whereas those of plants having the genotype (LmLm, CC) are rich in carvone (Hefendehl and Murray, 1973). Similarly, Maffei (1990) showed the effect of plant genotype on the composition of essential oils by crossing clone 7303 with M. spicata L.

Other strains are distinguished by the presence of eucalyptol (1,8-cineole) with a high rate in the strain of Marrakech (21.1%) followed by those of Tiznit and the strain S1 of Larache with rates of 12.3% and 11.2%, respectively. The strains of Houara, Settat, Errachidia, Dakhla and the wild strain S1 of Ifrane have rates that vary from 6.1% to 9%. On the other hand, the other strains have low rates (0.8% to 4%). Cook et al (2007), reported that the main constituents of the essential oil of spontaneous mint (M. spicata) from 3 locations on the island of Zakynthos were trans-piperitone oxide, piperinone oxide and 1,8-cineole.

Menthone is present in large quantities in strains of Errachidia (16.3%), Larache (S2) (15.1%), Agadir (14%) and Settat (12%). However, it is very low in strains of Houara, Tiznit, Dakhla and the wild strain S1 of Ifrane (0.1 to 1.5%), and it is absent in the other strains (Table 2).

3-cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)- is produced by the S1 strain of Larache and the two wild strains S1 and S3 of Ifrane with high levels of 22.1%, 20.5% and 19.7, respectively, whereas it is synthesized in small quantities or not in others (strains of Marrakech, Agadir, Errachidia and Ouarzazate). Contrary to strains S1, S2, S3, of Ifrane and S1 of Larache which are characterized by the absence of germacrene D, those of Tiznit, Larache (S2), Houara and Dakhla produce amounts ranging from 5.7 to 7.3%. On the other hand, the strains of Ouarzazate, Marrakech, Settat, Agadir and Errachidia have rates that vary from 2.5 to 4.2%.

With the exception of the strain of Houara, in which the linalool is present with a percentage of 5.4%, linalool levels in the other strains vary between 0 and 0.4%. This variation has also been observed by some authors. For example, Deschepper (2017) reports that linalool is generally absent or very weakly represented in the essential oil of spearmint. In contrast, wild mints from northern Greece have a very high level of linalool (85 to 94%) depending on the harvest season, unlike limonene and carvone which are only present in trace or absent (Kofidis et al., 2004). Menthol is only found in the strains of Agadir (Figure 3), Larache (S2) and Errachidia with levels of 11.6; 5.6 and 2.1%, respectively. Except that of Errachidia, they contain pulegone at high levels of about 30.66 and 11.02%, respectively. The presence of pulegone in the leaves of M. spicata was reported by Soilhi et al. (2019). Other studies have mentioned that the pulegone is the main compound of the essential oils of M. rotundifolia and M. longifolia (Fleisher and Fleisher, 1991; El Arch et al., 2003; Oyedeji and Afolayan, 2006; Gulluce et al., 2007; Mkaddem et al., 2009; Hajlaoui et al., 2010), suspected to be the ancestors of M. spicata (Gobert et al., 2002).

A cluster analysis was carried out to determine the similarity in composition of the 13 cultured or spontaneous strains according to the majority chemical compounds derived from their essential oils. The distances observed (> 26) on the dendrogram (Figure 4) show the presence of 3 distinct classes of strains:

• S1 and S3 of Ifrane and S1 of Larache rich in 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)- (Figure 4 and 5);

• S2 of Larache and the strain of Agadir rich in pulegone (Figure 4 and 5);

• S2 of Ifrane and strains of Ouarzazate, Marakech, Houara, Settat, Errachidia, Dakhla and Tiznit rich in carvone (Figure 4 and 5).

In addition, the examination of this last group shows the grouping of strains into relatively dissimilar subgroups (dissimilarity less than20) which are made up of strains of Haouara; Ifrane (S2) and Ouarzazate; Settat and Errachidia; Marakech; Dakhla and Tiznit (Figure 4).

The analysis of the links between the chemical compounds of the essential oils of the 8 strains of M. spicata of the last group (S2 from Ifrane, Ouarzazate, Marakech, Houara, Settat, Errachidia, Dakhla and Tiznit), showed that the essential oils of strains of:

Houara contains other compounds such as Cubebene <β-> (1.9%), Dihydro carveol <iso-> (0.7%), Jasmon <(Z)-> (1.3%), Humulene II epoxide (0.8%), in addition to its richness in carvone (Table 2).

Ouarzazate and S2 of Ifrane stand out in carvone with rates ranging from 72.25 to 65.94% (Table 2).

Although the Settat and Errachidia strains have carvone content ranging from 21.96 to 25.03%, they also contain higher l-Menthone levels which are around 11.95% and 16.27, respectively (Table 2).

Marrakech, Dakhla and Tiznit, although they contain carvone (43.7%; 30.15%; 26.13% respectively) and D-Limonene (11.21%; 12.47%; 13.71 % respectively), the Marrakech strain stands out with a high rate of eucalyptol (21.1%) and (-)-8-p-Menthen-2-yl, acetate, trans (3.39%) (Table 2).

These results obtained were confirmed by the PCA showing that the dispersion of the compounds in the plane formed by the two axes (F1 and F2) makes it possible to explain 57.25% of the total variability (Figure 6).

The first axis explains 34,12% of the total variation. It is positively correlated with 3-cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)-; piperitone oxide; cadinol <α->; 6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol; (-)-spathulenol, and negatively correlated with germacrene D. The second axis showed 23.13% of the total variance and a positive correlation with menthol and pulegone (Figure 6).

The dispersion of the 13 studied strains of M. spicata in the plane formed by axes (F1 and F2) shows that strains S1, S3 of Ifrane and S1 of Larache are characterized by the synthesis of compounds which are positively correlated with the axis F1, and the absence of germacrene D (Figure 3 and 5). The strains of Agadir and Larache (S2) stand out for the synthesis of menthol, pulegone and l-Menthone (Figures 6 and 7).

The present study revealed the existence in Morocco of new chemotypes of Mentha spicata characterized by their richness in eucalyptol (1.8 cineol), pulegone, carvone or

3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)-, such as the cultivated strains of Marrakech, Agadir, the spontaneous S2 of Ifrane, or the spontaneous S1 of Larache respectively.

As in our case, essential oils composition of M. spicata is known by a huge variation which constitutes a series of chemotypes described by several authors:

• Mentha spicata var. ‘Moroccan’ crispa group is represented by a single chemotype predominated by carvone (62.69-75.53%) followed by limonene (10.52-17.94%), transcarveol (0-5.22%) and dihydro-carveol cis (0.39-3.62%) (Soilhi et al., 2019);

• Chemotype rich in carvone (39.21–62.51%), 1.8-cineole (7.24–12.49%), limonene (6.07–18.45%), and dihydro-carveol cis (1.17–6.56%) (Soilhi et al., 2019).

• Chemotypes characterized by pulegone, carvone, linalool, piperitone, piperitone oxide, menthone/isomenthone, pulegone/menthone/isomenthone and pulegone/piperitone (Baser et al., 1999; Telci et al., 2004 and 2010);

• Four M. spicata chemotypes found in Greece characterized by the dominance of linalool, carvone/dihydrocarvone, piperitone oxide/piperitetone oxide and menthone/isomenthone /pulegone (Kofidis et al., 2004).

According to Kokkini and Vokou (1989), the existence of different chemotypes is a common feature of most Mentha species and hybrids. In this study, all the strains were acclimatized in the same environmental conditions. Therefore the diversity could be accentuated, in addition to the genome of the strains, to the environmental factors of the regions of origin. Indeed, Zhao et al. (2013) reported that the composition of the essential oils of Mentha haplocalyx, evolves differently according to the environmental factors of the origin environments.

CONCLUSION

The results of this study indicate huge differences in the yield and the composition of essential oils of cultivated or spontaneous strains of M. spicata from Morocco. Indeed the essential oil yield of cultivated or spontaneous M. spicata from Morocco is very high (1.32–5.83%) compared to Tunisia, China, Iran, Turkey and Greece (0.45–3%).

The present study also revealed the existence in Morocco of new chemotypes of M. spicata characterized by their richness in 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)-, eucalyptol, pulegone or carvone such as the cultivated strains of Marrakech, Agadir or Ouarzazate and the spontaneous S2 of Ifrane, respectively. However, there are one or only few chemotypes of M. spicata in other countries like for instance in China (one chemotype of carvone) or in Iran (two chemotypes of carvone or pulegone).

This characterization of essential oils variation is helpful in the development of M. spicata resources for pharmaceutical industries and fragrance. Further, these results can open research paths for the exploitation of wild strains in addition to the selection of cultivated strains that comply with the requirements of different fields of use of M. spicata.

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