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N.K. Patra · H. Tanveer · S.P.S. Khanuja
A.K. Shasany · H.P. Singh · V.R. Singh · S. Kumar

A unique interspecific hybrid spearmint clone with growth properties of *Mentha arvensis* L. and oil qualities of *Mentha spicata* L.

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Abstract Crossing blocks of *Mentha arvensis* cv Kalka (menthol mint without carvone) and *Mentha spicata* cv Neera (carvone mint without menthol) in alternate rows (2:1::Kalka:Neera), and pollination of florets of Kalka with that from Neera, yielded a carvone-rich variant among the open-pollinated seedlings. The variant possesses the hybrid phenotype, including the vigorous *M. arvensis* growth habit and the synthesis of rich oil aroma supplemented with a menthol tinge (carvone 64%–76% against 58% for the normal carvone mint cv Neera), and thus a novel combination of the essential oil. Chromosome counts and random amplified polymorphic DNA analysis confirmed that this spearmint variant, designated as the variety Neerkalka, is a unique interspecific hybrid (2n=5*x*=60) of *M. arvensis* cv Kalka (2n=8*x*=96) and *M. spicata* cv Neera (2n=2*x*=24). Vegetative multiplication of the hybrid was facilitated by its underground sucker-reproducing ability which is otherwise absent in spearmints. The per cent improvement in the variant ranged from 31–97 for herbage yield and 95–317 for oil yield over the standard spearmint varieties (MSS-5, Arka and Neera), with per hectare oil yields of 125.0 kg, 139.0 kg and 65.0 kg, respectively.

Keywords Spearmint · Interspecific hybridisation · Hybridity tests · Productivity analysis · Variety development

Introduction

Spearmint (*Mentha spicata* L.), being a major source of carvone-rich essential oil for perfumery and flavouring industries, is grown world wide. In India, its cultivation is sporadic, especially on account of its low biomass and oil yield and the general problem arising in its economic production of propagules. Unlike *Mentha arvensis*, which produces underground suckers whose small pieces can be used as propagules, already rooted branches (runners) must be used for the field planting of *M. spicata* (Ikedo and Udo 1954; Murray 1960, 1972; Janaki Ammal and Sobti 1962; Kovineva and Kodasa 1976; Kak and Kaul 1978, 1980, 1981; Korneva 1983; Nikolaev and Pisova 1988; Kukreja el al.,1991; Tyagi et al., 1992). While progressive genetic improvement has considerably increased the essential oil yield and menthol concentration of oil in *M. arvensis*, little effort has yet been made in the improvement of *M. spicata* for better production of carvone rich oil. Apparently, interspecific hybridisation between *M. arvensis* and *M. spicata* can be a means of incorporating the high essential oil-producing and the suckering ability of *M. arvensis* into *M. spicata* . Although an F_1 hybrid from a *M. arvensis* var. *piperascens* Holmes×*M. spicata* cross was successfully raised by Sobti (1962), apart from being only intermediate between the two parents in morphological characters it did not exhibit any improvement over either of the parents for the the mentioned economic characters. In the present paper, we describe the production and characteristics of an interspecific hybrid between *M. arvensis* cv Kalka and *M. spicata* cv Neera that possesses commercial viability.

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N.K. Patra (✉) · H. Tanveer · S.P.S. Khanuja · A.K. Shasany · H.P. Singh · V.R. Singh · S. Kumar
Central Institute of Medicinal and Aromatic plants, Field Station, P.O. Nagla Dairy Farm, Distt. U.S. Nagar, Uttar Pradesh – 263 149, India

Materials and methods

M. arvensiss cv Kalka (2n=8*x*=96) is dwarf, disease and pest-resistant, variety of Japanese or menthol mint, that yields highly menthol-rich essential oil. *M. spicata* cv Neera (2n=2*x*=24) is a variety of conventional spearmint. The major morphological and chemical markers in the two species were: the underground suckers, oval leaves and >80% menthol content in essential oil in the former species, and the absence of suckers, elongated leaves and carvone-rich (60%) essential oil in the latter species.

The breeding method involved intentional cross-pollination between *M. arvensis* cv Kalka and *M. spicata* cv Neera. Since the florets of both species are small in size, manual emasculation and pollination are extremely difficult to achieve. To overcome this problem of controlled pollination, the approach of developing hybrid seeds was modified towards increasing the incidence of natural outcrossing between restricted parents only. For this purpose, the selected plants were raised in alternate rows (2:1::Kalka:Neera) in the crossing block from the genetically pure suckers/runners maintained in the breeder's plot in isolation. The plants raised in this way were grown to flowering. The florets of *M. arvensis* were repeatedly dusted with manually collected pollen of *M. spicata*. Seeds were collected from *M. arvensis* (Kalka) and bulked. Similarly seed from *M. spicata* (Neera) florets were collected. The bulked seeds were sown separately in flat earthenware pots in November 1995. The seedlings that emerged were transplanted in the field and mature plants were compared with the two parental species in respect of morphology and essential-oil quality. The plant having leaves of intermediate shape and containing carvone-rich essential oil was selected for confirming its hybridity between the two parents using several morphological traits, chromosomal counts, DNA fingerprints and terpene profiles of essential oils. The plant habits, leaf shape and sizes, and the underground suckering of the hybrid and parent varieties were then compared. For the study of chromosomes, young flower buds of appropriate size in the three genotypes (the hybrid and the two parents) were fixed in Carnoy's solution (6:3:1) overnight and smear preparations of anthers from the fixed buds were made in 1% acetocarmine. Chromosome counting was carried out at the diplotene and diakinesis stages of 30 analysable pollen mother cells (PMCs) for each genotype. RAPD profiles were determined for the spearmint hybrid and the two parents to further confirm the ancestry of the parents of the hybrid at the DNA level. For this purpose total DNA was extracted from 40 mg of leaf tissue following the method described by Doyle and Doyle (1987). The amplification of random DNA sequences by the polymerase chain reaction (PCR) using the 12 random primers, designated as MAP01 to MAP12 (AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATTCCG, CCCTGCAGGC and CCAAGCTTGC, respectively), was performed according to the protocol reported by Williams et al. (1990). After all 40 PCR cycles were completed 10 µl of each sample were loaded on the 1.2% agrose gels containing 0.4 µg ml^{-1} of ethidium bromide for staining and subjected to electrophoresis in running buffer (89 mM Tris borate, pH 8.3, 2 mM of EDTA) at 10 V/cm. The bands were analysed using image master 1D elite software (Pharmacia) and the graphic phenogram of the genetic relatedness among the three genotypes was produced by means of UPGMA (unweighted pair group method with arithmetic averages) cluster analysis. The bands were photographed by a polaroid system (Millan et al. 1996).

Essential-oil content in the green herbage of the hybrid and the parents was estimated in Clavenger's apparatus. The terpene profiles of the essential oils of the three genotypes were determined by gas liquid chromatograhy (GLC) using 2 m×3 mm stainless columns packed with 5% OV-351 on an 80/100 supelcoport. A temperature programme of 80 to 130°C with a rise of 4°C/min was employed. The injector/detector temperature was maintained at 200°C. Data processing was done on a HP-3390 A integrator. For arriving at correct inferences about the differences among the genotypes in their mean performances for morpho-metric traits, the following standard statistical technique for computing LSD was used:

S.E. of the difference between the two variety means=$\sqrt{2V_E/r}$, where "V_E"=Error Mean Sum of Square and "r"=no. of replications, LSD=(S.E.) difference x $t_{5\%}$ (L.S.)

Table 1 Some of the distinguishing characteristics of the mint varieties Neera, Kalka, MCAS-2 and Neerkalka (number of single plants, N=60 per variety)

Traits	Hybrid Neerkalka (mean±SE)	*M.spicata* cv Neera (mean±SE)	*M. arvensis* cv Kalka (mean±SE)	*M. cardiaca* cv MCAS-2 (mean±SE)
Plant height (cm)	56.8±2.1	52.4±2.1	97.0±2.2	48.6±2.2
Leaf length (cm)	7.4±0.4	2.3±0.2	9.9±0.4	6.3±0.3
Leaf width (cm)	2.7±0.2	0.8±0.1	2.7±0.1	1.1±0.1
Leaf colour[a]	Green (137A)	Green (137B)	Green (137C)	Green (137B)
Leaf shape	Elliptical to ovate	Lanceolate	Elliptical to ovate	Elliptical to ovate
Leaf margin	Finely serrated	Normal serration	Normal serration	Finely sharp serration
Leaf veins	Highly prominent	Distinct	Much distinct	Much distinct
Stem colour[a]	Green (144C) with purple pigments at the base(186C)	Green (144C) with purple pigments at the base (186D)	Green (144D) with purple pigments at the base (186C)	Green (144C) with purple pigments at the base (186C)
Inflorescence	Racemose of axillary verticillasters	Indefinite racemose	Racemose of axillary verticillaster	Racemose of axillary verticillaster
Flower colour[a]	Whitish (69D) purple (73B)	Whitish (69D)	Whitish (69D)	Whitish (69D) purple (73B)
Single plant weight (g)	184.4±2.1	73.4±3.3	208.0±4.8	172.4±3.1
Leaf:Stem	0.61±0.03	0.54±0.01	0.90±0.01	0.58±0.02
Herbage yield q/hectare)	128	80	165	94
Oil content (%)	0.80	0.60	0.80	0.64
Oil yield (kg/hectare)	102.4	48.0	132.0	60.2
Oil aroma	Carvone based with menthol tinge	Carvone based	Menthol based	Carvone based
Growth habit	Semi-prostrate	Prostrate	Erect	Erect

[a] Colour codes are according to the RHS colour chart published by the Royal Horticultural Society, 80 Vincent Square, London SW1P2PE, 1995; 1,2 data obtained in preliminary yield trial; S.E.=standard error of mean = S/√N (where S=standard deviation of the sample size N)

Fig. 1A–C Morphology of the hybrid Neerkalka. **A** Racemose inflorescence of axillary verticillasters; **B** plants with elliptical to ovate leaves; **C** profusely developed underground suckers in the hybrid (*NK*), compared to the parents cv Kalka (*K*) with relatively less suckers and cv Neera having no suckers (*N*)

Results and discussion

Two hundred and sixty and two hundred and ninety seedlings were produced by the seeds collected from the florets of *M. arvensis* cv Kalka and *M. spicata* cv Neera, respectively. The seedlings from *M. spicata* were almost alike in morphology to the parental phenotype (Neera) and possessed a similar carvone-rich smell. All the seedlings from the *M. arvensis*, except one with an unusually carvone-rich oil aroma and a profuse sucker forming habit (Fig. 1), resembled the morphology of *M. arvensis*. The plants of *M. arvensis* cv Kalka and the putative hybrid (variant) were subsequently raised in large numbers from their pure suckers, and those of *M. spicata* cv Neera using aerial small runners and rooted branches, from nearby mother plants in January 1996 for their detailed comparison (Table 1). The plants of the hybrid-looking genotype were similar to those of *M. arvensis* cv Kalka in possessing a vigorous growth habit, long smooth leaves with prominent veins, and underground suckers. They shared with *M. spicata* cv Neera plants a prostrate canopy, finely serrated leaf margins, and a carvone-rich shoot aroma. Such co-expression of the characters of the two species, and especially the expression of the major marker character (the carvone-rich oil aroma) of the pollen parent in the variant, gave a clear indication that it was indeed a hybrid resulting from effective pollination between the two parental species. A comparison of plants of the hybrid genotype with the plants of a *Mentha cadiaca* (Scotch mint) genotype available to us, showed that the variant shared some of the morpho-physiological attributes, including a carvone-rich aroma, with *M. cardiaca*.

The results of chromosome counts revealed that the variant had 2n=60, which was in agreement with the sum of the chromosome numbers of *M. arvensis* cv Kalka (n=48) and *M. spicata* cv Neera (n=12) (Fig. 2). The variant was thus confirmed to be an allopentaploid hybrid, with 2n=5*x*=60, between the two parental species. It is worthwhile mentioning that, in contrast to the earlier belief of the nonexistence of other ploidy levels than tetraploidy in the species *M. spicata* (Harley and Brightom 1977), the variety Neera affiliated to this species, as revealed by this study, is not a tetraploid (2n=48) but a diploid with 2n=24. Accordingly, the present hybrid can be catalogued as a new hybrid having the chromosome number 2n=60 as against the earlier report of a hexaploid hybrid (2n=72) between these two species (Sobti 1962). To differentiate it from the earlier hybrid we have designated it by the name Neerkalka.

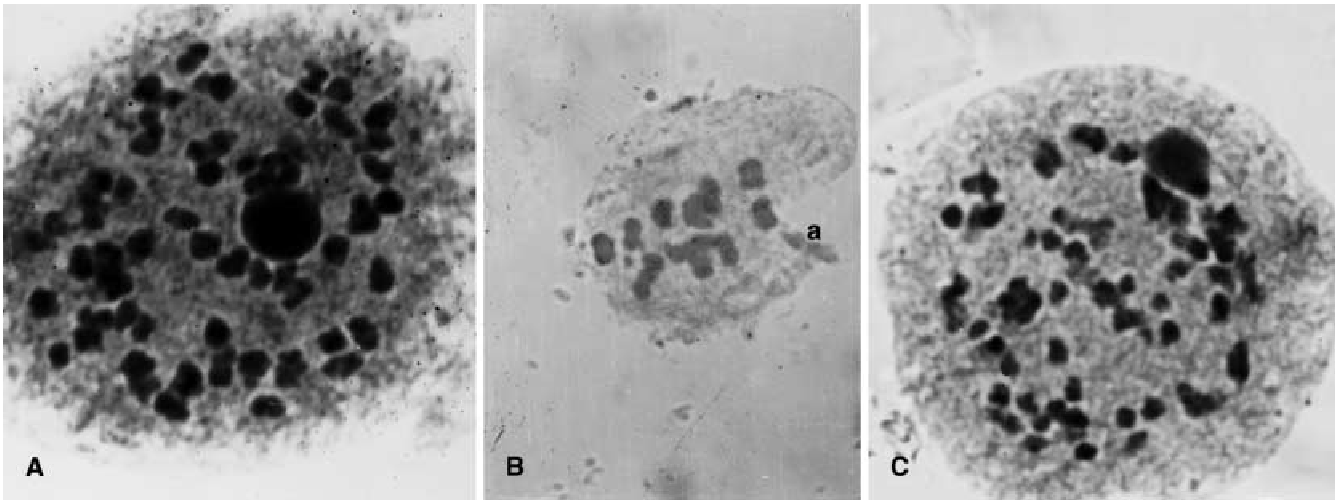

Fig. 2A–C Chromosomal ploidy status in the hybrid and the parents. **A** A PMC of the hybrid at the diakinesis phase with 2n=5*x*=60; **B** a PMC of the pollen parent Neera, exhibiting 2n=2*x*=24 (12 bivalents) at meiotic first metaphase; **C** a PMC of the female parent Kalka at the diakinesis phase with 2n=8*x*=96). *a*=artefact

Table 2 Important oil constituents in the hybrid Neerkalka

Constituents	% Total oil
Limonene	6.8–23.20
Menthol	0.7–2.5
Carvone	64.0–76.1

Table 3 Comparative yield performances of the hybrid Neerkalka and three popular varieties, MSS-5, Arka and Neera, of *M. spicata* in the 1997–1998 pilot scale over two consecutive season harvests. The break-up of annual oil yield /plot for the two harvests in each case shown in parenthesis

Name of the variety	Herb yield/plot (16 m×5 m) (kg)			Annual herb yield/ ha (q)	Oil content (%)		Annual oil yield/plot (kg)	Annual oil yield/ ha (kg)	Per cent improvement in Neerkalka over the other varieties for	
	First harvest	Second harvest	Total (kg)		First harvest	Second harvest			Herb yield	Oil yield
MSS-5	62.50	116.60	179.10	224.00	0.62	0.50	1.00 (0.40+0.60)	125.00	40.20	117.00
Arka	66.35	124.40	191.00	239.00	0.65	0.55	1.11 (0.43+0.68)	139.00	31.40	95.14
Neera	48.00	79.00	127.00	159.00	0.45	0.36	0.52 (0.22+0.30)	65.00	97.50	317.31
Neerkalka	82.43	168.40	251.00	314.00	1.00	0.80	2.17 (0.82+1.35)	271.25	–	–
LSD (5%)	4.16	9.52	14.00	17.5	0.10	0.10	0.20	12.5	–	–

Our further confirmation of the hybridity of Neerkalka was provided by RAPD analysis. The profiles of the amplified products from the two parental species were clearly different from each other. Neerkalka possessed common fragments of both parents, confirming that it contained co-dominantly expressed genomes of both parents (Fig. 2A and B).

RAPD analysis of *M. cadiaca* demonstrated large differences with Neerkalka (Fig. 2B). It was thus confirmed that Neerkalka, despite its apparent similarity with *M. cardiaca* for some morpho-physiological plant attributes, did not have a close genomic relationship with the latter species.

That Neerkalka, despite its similarity with the pollen parent *M. spicata* cv Neera for its carvone-rich oil aroma, is distinct from both parents for its chemical oil constituents was revealed by the GLC results of the essential oil. The oil of the female parent *M. arvensis* cv Kalka contained 80–82% menthol but no carvone or carveol, while that of the pollen parent *M. spicata* cv Neera contained 58% carvone but no menthol, menthone or menthyl acetate. The hybrid, in contrast to the parents, contained 64–76% carvone and 0.66–2.45% menthol depending upon various stages of growth (Table 2). The essential oil with such a high carvone content supplemented with the menthol tinge led Neerkalka to excel over its parents for its novel pleasant smell.

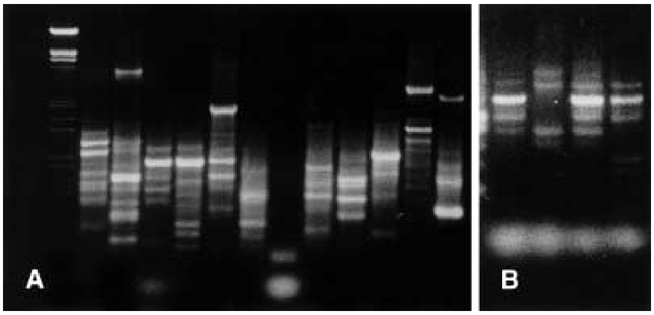

Fig. 3A, B RAPD analysis of the hybrid and the parents. **A** Polymorphic DNA patterns of the hybrid; *lane 1* Marker: *lanes 2 to 13* RAPD patterns with MAP 01 to MAP 12. **B** Co-dominance of RAPD bands with MAP 03; *lane 1* Kalka; *lane 2* Neera; *lane 3* Neerkalka; and *lane 4 M. cardiaca*

In order to determine the economic viability of Neerkalka, the variety was assessed during 1997–1998 for its biomass and oil productivity against the existing standard cultivars, MSS-5, Arka and Neera (the pollen parent), of *M. spicata*. As revealed by the results of a yield trial at the commercial pilot scale, the per cent improvement in Neerkalka over the three standard cultivars, MSS-5, Arka and Neera, with a 125.0 kg, a 139.0 kg and a 65.0 kg oil yield/hectare, respectively, ranged from 31–97% for herbage (green biomass) yield and 95–317% for oil yield (Table 3). Neerkalka, besides registering such a remarkably high yield, exhibited resistance to leaf spot and rust diseases when planted in the infected field. Only 0–4 plants in a field of 2000 were observed to develop these diseases in separate trials. The disease-resistant characters were apparently inherited from the parent cv Kalka. Since its isolation in 1994, the hybrid has shown stability in its morphological characters, its herbage and its oil yield over five mint-growing seasons. The genotype has aggresive suckers growing under the soil surface to provide protection from adverse weather conditions and mechanical damage. The plant with all its morpho-physiological characters firmly fixed, is propagated vegetatively and a large amount of planting materials (suckers) became available in a short period of time, as compared to the time needed for the existing normal spearmint cultivars having no suckers but only aerial small runners. This new hybrid has been released recently for its commercial cultivation in farmer's fields and its economics is attracting crop growers very rapidly (Anonymous 1998).

To summarize, in this study we succeeded in producing a much-beneficial interspecific spearmint hybrid, with a new chromosome number ($2n=5x=60$) and a unique RAPD profile, between *M. arvensis* cv Kalka (the female parent with $2n=8x=96$) and *M. spicata* cv Neera (the pollen parent with $2n=2x=24$). This was achieved by enhancing natural outcrossing by the judicious planting of the two minute-flowered species *M. arvensis* cv Kalka and *M. spicata* cv Neera in alternate rows (2:1::Kalka: Neera) in experimental crossing blocks and regular dusting the non-emasculated florets of Kalka with the pollen of Neera. The hybrid, designated as Neerkalka, resembles the *M. arvensis* growth habit with a very pleasant aroma, accredited to the novel combination between the very high carvone and the menthol tinge in its essential oil. Apart from its high quality oil, a high oil productivity, and its adaptive advantage due to underground suckers distinctly mark Neerkalka from all the existing normal spearmint varieties. These results showed that interspecific hybrid plants with a novel flavour and a much-upgraded oil productivity could be produced in even minute-flowered species having the problem of manual emasculation, by adopting strategic enhancement of their outcrossing and progeny selections mainly based on male-marker traits. In considering the results of the oil composition in the hybrid and the parents it appears that all of its oil components, except the negligible menthol fraction, were contributed by the male parent *M. spicata* cv Neera. This is in agreement with the earlier finding of Nikolaev and Pisova (1988). On the basis of interspecific crosses these authors amply demonstrated that the oil composition of mint hybrids generally depends on the terpenoid composition of the pollen parent. The present study has also revealed a considerable morpho-physiological similarity of Neerkalka with the other cultivated carvone-rich mint species *M. cardiaca* (Scotch mint) which is thought to be a natural hybrid of the same parental species, *M. arvensis* and *M. spicata* (Schultz 1854; Lincoln et al. 1986). Even so, our biochemical results have revealed that the RAPD patterns of Neerkalka are very diffferent from those of *M. cardiaca*. Taken together, and given equal importance, these two sorts of analyses led us to suggest the possibility that in the course of evolution both of the ancestral species of *M. cardiaca* had undergone significant genome re-patterning which might not have much affected the co-adapted linked genes responsible for their morpho-physiological fitness. However, present day cultivated forms (like cv Kalka and cv Neera) with such genomic re-patterning, when involved in cross hybridisation, have resulted in a hybrid (like Neerkalka) with a RAPD pattern, completely different from that of the ancient hybrid *M. cardiaca*.

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