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Molecular analysis reveals that lack of chasmothecia formation in *Erysiphe necator* in Maharashtra, India is due to presence of only *MAT1-2* mating type idiomorph

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Summary

The heterothallic, ubiquitous fungus *Erysiphe necator* causes powdery mildew disease of grapevines and in many countries it is reported to reproduce both asexually and sexually. Sexual reproduction results in the formation of chasmothecia (cleistothecia) on infected surfaces when the colonies of two opposite mating types meet on the infected plant parts and the temperatures are favorable for their development. Chasmothecia are reported from north India, but not from peninsular India, even though powdery mildew is reportedly present in these regions at least since the beginning of the last century. Through systematic survey of ten vineyards in Maharashtra and adjoining Karnataka in peninsular India, we confirmed the absence of chasmothecia under natural conditions. Analysis of temperature data from two locations in Maharashtra showed that the *T*max and *T*min were favourable for chasmothecia initiation (10 °C to 30 °C) and maturation (15 °C to 30 °C) for a sufficient period of time.

Multiplex PCR of 120 *E. necator* field isolates collected from peninsular India showed presence of a single band of 232 bp corresponding to *MAT1-2* mating type idiomorph or MAT- phenotype. None of the samples gave band of 408 bp corresponding to *MAT1-1* mating type idiomorph. Further, two bands of 408 bp and 232 bp were detected in only one powdery mildew sample collected from Kashmir, in north India while the other nineteen samples gave a single band of 232 bp. Thus, molecular analysis established that *E. necator* is not sexually reproducing in Maharashtra due to presence of only one mating type idiomorph. The study also brings out that *MAT1-1* mating type idiomorph is not as common in nature as *MAT1-2* and explains why in many other countries, too, chasmothecia were first observed as late as half to one century after start of grape cultivation.

K e y w o r d s : powdery mildew; grape; MAT+ phenotype; *MAT1-1* mating type idiomorph.

Introduction

Grapevine powdery mildew caused by the obligate, ascomycete pathogen, *Erysiphe necator* (Schw.) (syn. *Uncinula necator* [Schw.] Burr.; anamorph *Oidium tuckeri*), is a widespread and destructive disease of grapevine in the world. It is reportedly present in Maharashtra, India at least since the beginning of the last century (Uppal *et al.* 1931) and apart from reducing vine vigour and assimilation of photosynthates, it affects fruit quality and shelf-life (Sawant and Sawant 2012). In temperate viticultural areas, *E. necator* reproduces asexually during active vine growth and sexual reproduction occurs when the vines start entering into low temperature induced dormant stage. It results in formation of fruiting bodies known as chasmothecia (formerly known as cleistothecia) which help the fungus to overwinter. *E. necator* is a heterothallic fungus and when the two mating type idiomorphs are present, chasmothecia can form at 20 °C but are inhibited above 30 °C or below 10 °C temperature (Gadoury *et al*. 1988, Legler *et al*. 2012) and by high precipitation (Diehl and Heintz 1987). Chasmothecia of *E. necator* are reported from Kashmir (Putto and Razdan 1987) and Punjab (Munshi *et al*. 1996) in north India where vines undergo dormancy, but till date there is no report about their presence on grapevines in Maharashtra, in peninsular India, where the grapevines remain ever-growing without a dormant period. In Europe and many warm tropical countries like Australia, Taiwan, South Africa and Peru, *E. necator* chasmothecia were first observed 50 to 100 years after introduction of powdery mildew in that country (Wicks and Magarey 1985, Kuo *et al.* 1989, Hallen and Golz 2000, Bendezú-Euribe and Alvarez 2012). It was presumed that only one mating type idiomorph might have been present initially and the late introduction of the opposite mating type idiomorph led to the initiation of the sexual reproductive cycle.

This study was undertaken to investigate whether chasmothecia of *E. necator* were initiated but not maturing due to adverse environmental conditions in Maharashtra, India or whether there was absence of sexual reproduction due to presence of only one mating type idiomorph.

Material and Methods

Research, commercial and germplasm blocks, situated in Pune, Nasik and Bijapur, planted with 'Thompson Seedless', imported table and wine grape cvs., and about 400 grape accessions were monitored for chasmothecia formation from 2011 to 2014. Randomly collected infected leaves were observed microscopically for presence of ascocarps. Temperature and RH data recorded on automatic weather station (µMetos) from 2002 to 2013 was retrieved and computed to get weekly and monthly averages of

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maximum (*T*max) and minimum (*T*min). One hundred and twenty *E. necator* samples from 31 vineyards in Maharashtra and Karnataka and 20 samples from Srinagar, Kashmir were collected during 2013-14.

Before analysis, *E. necator* was cultured on healthy, surface sterilized leaves of 'Thompson Seedless'. To increase the chances of detection of the two idiomorphs, care was taken to harvest conidia from five to six lesions from each sample. Dishes containing the inoculated leaves were incubated with a 12 h photoperiod at 25 °C for 12 d in a growth chamber (Binder KBWF 720).

The culture was used for detection of *MAT1-1* and *MAT1*-2 idiomorphs following the method of BREWER *et al*. (2011). DNA was extracted by using chelex 100 resins (Bio-Rad). DNA from some of the field samples with abundant fungal growth was directly extracted using Qiagen Plant Mini DNA extraction Kit. PCR was carried out using primer pairs EnαF2 and EnαR3 for *MAT1-1*, EnHMGF1 and EnHMGR1 for *MAT1-2*. PCR reaction was carried out in a final volume of 25 µL, containing 10x PCR buffer 2.5 µL (Merck), 2.5 µL dNTPs (2.5 mM each), 1.0 µL each of 10 µM primers, 0.75 U *Taq* polymerase enzyme (Merck), and 1 µL DNA template. PCRs were run on an ABI Gold Geneamp PCR System 9700 for 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s with an initial denaturation of 2 min at 95 °C before cycling and final extension of 5 min at 72 °C after cycling. The 20 cultures from Kashmir and twenty randomly selected cultures from Pune were further analysed by singleplex PCR using primer pairs EnαF2 and EnαR3. PCR products were resolved in 1.2 % Agarose gels, stained with ethidium bromide and visualized using gel-documentation system (Alpha Ease FC™version 4.0.1, Alpha Innotech Corporation).

Results and Discussion

Chasmothecia were not observed in field at any location even though the highly susceptible accessions were severely infected with *E. necator*. Microscopic observations showed only surface mycelial growth with conidiophores and chains of conidia. Weekly averages of *T*min and *T*max for the year 2013 were between the favourable range of 10-30 °C from 11th June to 23rd September in Pune, while at Nasik the temperatures were favourable for most part of the year except from a brief period from 19th February to 11th June. Previous 12 years average weather data from Pune showed that the monthly *T*min was above 10 °C throughout the year, while *T*max was below or about 32 °C from July to October and from November to January and the RH was also within the required range (Fig. 1). Thus, we confirmed the absence of chasmothecia under natural conditions in Maharashtra even though powdery mildew build up was for sufficient duration to allow pairing of opposite mating types and temperatures and RH were also in favourable range (GADOURY *et al*. 1988). Chasmothecia were not observed in Kashmir, too, and only one grower reported formation of chasmothecia in his vineyard in some years but not every year. Powdery mildew infection was seen in only four of the eighteen vines observed in residential areas in Kashmir but there was no history of chasmothecia formation in any of these vines. In multiplex PCR, only one *E. necator* culture from a commercial vineyard in Kashmir gave two bands of approximately 232 bp and 408 bp showing presence of both mating type idiomorphs showing presence of both *MAT1*-2 and *MAT1-1* idiomorphs (Fig. 2a). This culture originated from the vineyard which had previous history of chasmothecia formation. The other 19 cultures gave a single band of 232 bp corresponding

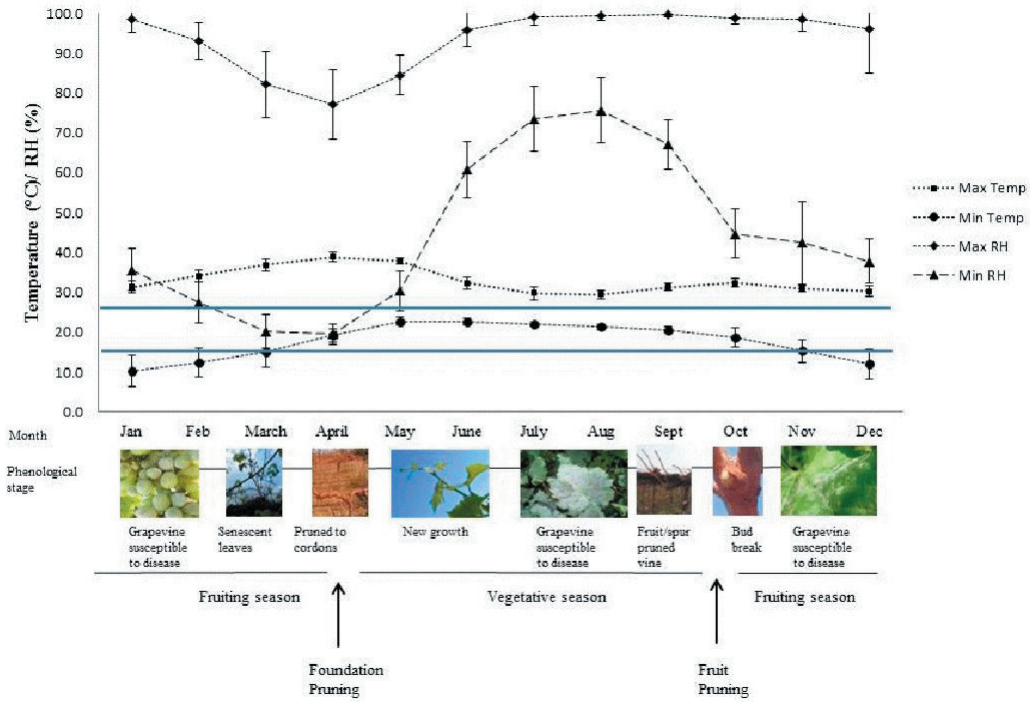

Fig. 1: Mean of twelve years monthly averages of maximum and minimum temperature and relative humidity at Pune, Maharashtra, India and corresponding vine growth stages.

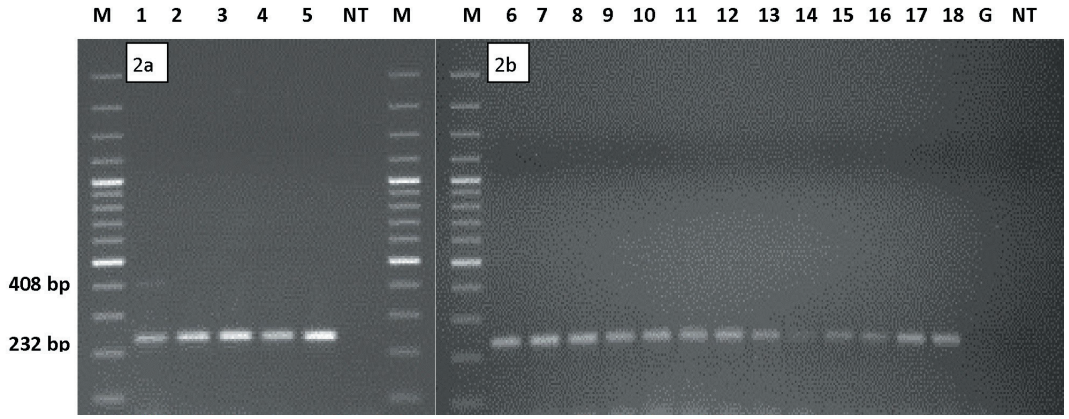

Fig. 2: (**a**) and (**b**), Electrophoretic pattern showing the PCR product from DNA extracted from isolates of *E. necator* collected from different locations in India. Kashmir (lanes 1 to 5); Maharashtra, lanes 6 to16; Karnataka, lanes 17 & 18; G denotes grape DNA, NT denotes non-template, and M denotes 100 bp molecular weight marker. PCR products of 232 bp and 408 bp correspond to *MAT1-2* idiomorph and *MAT1-1* idiomorph respectively.

to *MAT1-2* idiomorph. The 120 cultures from Maharashtra and Karnataka gave a single band at approximately 232 bp corresponding to *MAT1-2* mating type idiomorph (MAT- phenotype) (Fig. 2b). None of the samples gave band of 408 bp which corresponds to *MAT1-1* mating type idiomorph (MAT+ phenotype). As both mating type idiomorphs are reported to be equally common (Miazzi *et al*. 1997) and present in almost 1:1 ratio, the chances for non-detection of *MAT1-1* idiomorph were negligible. Earlier, Delye *et al*. (1997), too, had shown presence of only MAT- phenotype in Indian *E. necator* isolates on the basis of an isolate pairing study. In singleplex PCR, too, the positive culture from Kashmir gave a band of 408 bp specific for *MAT1-1* idiomorph while the remaining 19 cultures from Kashmir and other 20 cultures from Maharashtra did not produce any PCR product (gel image not shown). Results show that even in Kashmir, the *MAT1-1* idiomorph is not as prevalent as the *MAT1-2* idiomorph and explains why chasmothecia are sighted sparingly and sporadically, even 25 years after they were first reported by Putto and Razdan (1987).

It is intriguing to note that while in many countries both mating types are reported to be equally present under natural conditions, in Kashmir even in the long period of at least 20-25 years, the *MAT1-1* idiomorph is not able to establish itself as effectively as the *MAT1-2* idiomorph. Our results lend credence to the presumption that in countries where chasmothecia were reportedly sighted after 50 to 100 years of introduction of the disease, only one mating type idiomorph was present for such a prolonged period.

To the best of our knowledge, there is no documentary evidence to show which mating type idiomorph was in existence in these countries prior to the sighting of chasmothecia. Our results show that it is the *MAT1-2* idiomorph which is well established in vineyards in Peninsular India, and it is quite possible that in the other countries, too, the same idiomorph might have been present initially. This can only be confirmed by molecular analysis of *E. necator* population from other geographical areas where chasmothecia have not been observed so far. Sexual reproduction provides more chances of genetic recombination which results in development of new strains with attributes like enhanced fungicide resistance (Diehl and Heintz 1987) and spread of fungicide resistance genes (Stummer *et al* 2003), which may further compound the present difficulties in managing this disease in Maharashtra. Hence it is important to prevent the entry of *MAT1-1* idiomorph in this region.

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