

ECOLOGICAL AND GENETIC ADVANCES IN THE CULTIVATION OF *TUBER* SPP.AVANCES ECOLÓGICOS Y GENÉTICOS EN EL CULTIVO DE *TUBER* SPP.Alessandra Zambonelli^{1*}, Pamela Leonardi¹, Mirco Iotti² and Ian Hall³

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SUMMARY

Recent advances on the ecology and genetics of true truffles (*Tuber* spp.) are reported and their impact on truffle cultivation is discussed. New insights have been gained on truffle soil ecology and interrelationships of truffles with associated microorganisms in the soil. For instance, some bacteria seem to play a key role in truffle fruiting body formation and maturation. However, the most important advance in truffle genetics over the past 20 years has been the sequencing of the *Tuber melanosporum* genome and the discovery that truffles, like other Pezizalean fungi, are heterothallic. This finding has had a significant impact on research on truffles and many studies have been devoted to better understanding the distribution of the mating types in soil in natural and cultivated truffières. The characterization of the mating type idiomorphs of several *Tuber* species has led to the possibility of selecting mycelial strains for truffle cultivation in particular sites.

Index words: True truffles, ecology, genetics, cultivation.

RESUMEN

Avances recientes en la ecología y la genética de las trufas verdaderas (*Tuber* spp.) se reportan y se discute su impacto sobre el cultivo de la trufa. Nuevas perspectivas han surgido en la ecología del suelo de la trufa y las interrelaciones de las trufas con microorganismos asociados en el suelo. Por ejemplo, algunas bacterias parecen tener un papel clave en la formación del cuerpo fructífero de trufa y su maduración. Sin embargo, el avance más importante en la genética de la trufa en los últimos 20 años ha sido la secuenciación del genoma de *Tuber melanosporum* y el descubrimiento que la trufa, como otros hongos Pezizalean, es heterotética. Este hallazgo ha tenido un impacto significativo en la investigación sobre trufas y muchos estudios se han dedicado a entender mejor la distribución de los tipos de apareamiento en el suelo en truffières naturales y cultivadas. La caracterización de los idiomorfos del tipo de apareamiento de varias especies *Tuber* ha conducido a la posibilidad de seleccionar cepas miceliales para el cultivo de trufa en sitios específicos.

Palabras clave: Trufas verdaderas, ecología, genética, cultivo.

INTRODUCTION

True truffles (*Tuber* spp.) are the most valuable of the edible ectomycorrhizal mushrooms. They are characterized by hypogeous fruiting bodies, many of which have evolved intense aromas for dispersing their sequestate spores by animals (Trappe and Claridge, 2010). Their unique aromasmake these fungi unique in European cuisine.

The most economically important species of truffle, *Tuber magnatum* Pico (Italian white truffle), *Tuber melanosporum* Vittad. (Périgord black truffle), *Tuber aestivum* Vittad. (Summer truffle or Burgundy truffle) and *Tuber borchii* Vittad. (bianchetto truffle), naturally grow only in Europe, and Italy and France are the countries with the longest tradition of their consumption. The gastronomic interest in truffles and their economic value has stimulated researchers to find the most efficient methods for cultivating them. The first Italian and French attempts to cultivate truffles dates to the Renaissance. However, the first real success did not start until the early 1800s when Josef Talon in France developed a crude but effective method for cultivating the Périgord black truffle (Hall et al., 2007; Hall and Zambonelli, 2012).

Modern truffle cultivation began in the early 1970's when Italian and French researchers improved techniques to produce plants from seedlings or cuttings colonized by the target species of truffle (Zambonelli et al., 2015). Around the same time the first studies on *Tuber* ecology were being carried out with the aim of defining the ideal soil and climatic conditions suited to the various species of truffle (Delmas and Durand, 1971; Montacchini et al., 1977; Zambonelli and Di Munno, 1992). These studies established the basis for the explosion of truffle cultivation in France and Italy and soon after in Spain (Reyna and Garcia-Barreda, 2014). Beginning in the 1980s, *Tuber* cultivation was introduced into other European countries where truffles were not traditionally appreciated, and many non-European countries including Argentina, Australia, Chile, China, Israel, Morocco, New Zealand, South Africa, and USA also got involved (Berch and Bonito, 2014; Hall and Haslam, 2013; Wang, 2012; Reyna and Garcia Barreda, 2014; Zambonelli et al., 2015).

Since the turn of the century significant advances on *Tuber* genetics and molecular ecology were achieved with the contributions of researchers in several European countries and particularly the USA and Australia. Moreover, a suite of molecular approaches is now available to commercial

nurseries and truffle growers either to check the quality of inoculated plants or to evaluate the status of natural and cultivated truffières (for a review see Parladé *et al.*, 2016). In this review, we focus our attention on recent major advances on truffle ecology and our understanding of the genetics of the genus *Tuber*, which are likely to have a major bearing on their future cultivation.

ECOLOGICAL STUDIES

Most of the ecological studies carried out over the past 30 years have regarded the abiotic environment where the truffle lives, and the physical and soil chemical characteristics, and climate. Relatively few studies have paid attention to the flora, fauna and vegetation of the truffle producing areas (Zambonelli and Di Munno, 1992). This has been a mistake because truffles live in complex environments and intimate relationships with other soil organisms including other fungi, as well as bacteria and invertebrates which may influence the survival of truffle mycelium and its ability to colonize suitable host plants (Figure 1). These in turn may trigger fruiting or modify the organoleptic qualities of truffles.

Attention has been aimed at other ectomycorrhizal (ECM) fungi living on the same plants hosting the mycorrhizas of *Tuber* in cultivated and in natural truffières (De Miguel *et al.*, 2014). The ECM fungi were generally assumed to compete with *Tuber* spp. in a sort of underground warfare aimed at mycorrhizal domination (Hall *et al.*, 2007). This concept might well be visualized in a young truffle orchard where seedlings are (hopefully) only colonized by

Tuber spp. but then quite quickly also became colonized by the native ECM fungi. In this situation only if the soil characteristics are suitable will the introduced *Tuber* species would be able to compete with other ECM fungi for space on the young roots.

When soil conditions are suitable to the truffle development, competition may occur between different *Tuber* species. For example, *T. melanosporum* was found to compete with *Tuber brumale* Vittad. in most of the countries where truffle cultivation has been introduced (Berch and Bonito, 2014; Guerin-Laguette *et al.*, 2013; Linde and Selmes, 2012; Reyna, 2012; Souzart, 2011). In Israel and southern Italy the most competitive truffle with *T. melanosporum* seems to be *T. aestivum* (Belfiori *et al.*, 2012; Bencivenga *et al.*, 1992; Turgeman *et al.*, 2012). Similarly, in New Zealand problems of competition have also been found between *T. borchii* and *Tuber dryophilum* Tul. & C. Tul. and between *T. melanosporum* and *Tuber maculatum* Vittad. (Guerin-Laguette *et al.*, 2013; Hall, personal communication; Hall *et al.*, 2007). The problems of *Tuber* spp. contamination in cultivated truffières has been much more serious when the origin of these contaminations has been the nursery.

There is the risk of introducing unwanted *Tuber* species which may be invasive and may replace native or more valuable European truffle species. For instance, the Chinese black truffle, *Tuber indicum* Cooke & Massee, has already been found to contaminate plants inoculated with *T. melanosporum* in Italy (Murat *et al.*, 2008). Although the Chinese species *Tuber sinoestivum* J. P. Zhang & P.G. Liu (Zhang *et al.*, 2012) has not yet been found to contaminate *T. aestivum* inoculated plants in Europe, there is the ever present risk that this species will find its way onto European plants either through the accidental or deliberate mixing of its spores with *T. aestivum* in inocula (Zambonelli *et al.*, 2012). To reduce the risks of having contaminated inocula used to produce *Tuber* mycorrhized plants, truffles can be screened and incorrect species removed prior to the production of inocula. Legislation on the certification of mycorrhized plants has also been applied in some countries, but is not universal in Europe (Andrés-Alpuente *et al.*, 2014; Murat, 2015).

The situation in natural truffières is quite different from plantations. In a mature forest, a root system is colonized by different ECM fungi which establish a dynamic equilibrium and are dependent on the soil's biotic and abiotic conditions. Distinct species of *Tuber* can share the same soil niches or occupy distinct soil patches. For example, Leonardi *et al.* (2013) found ectomycorrhizas of different *Tuber* species close to *T. magnatum* ascomata whereas *T. borchii* and *T. dryophilum* were never found to colonize the same patch of soil (Iotti *et al.*, 2010).

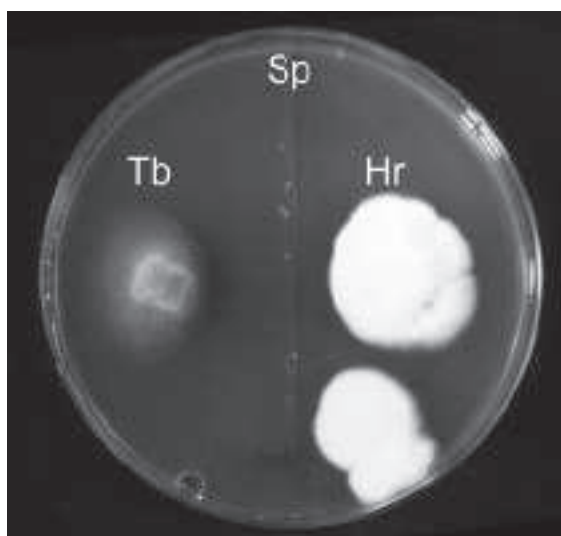


Figure 1. Effect on mycelial radial growth of the co-inoculation of *Tuber borchii* (Tb) and *Hebeloma radicosum* (Hs) with *Staphylococcus pasturii* (Sp). The bacterium strongly inhibits only the mycelial growth of *T. borchii*.

At the end of the 20th century the ECM fungal communities in truffières were studied using morphological techniques (De Miguel *et al.*, 2014). In these studies, root samples were first examined under a stereomicroscope and their external features described. A small portion of the mantle was then observed under a light or interference microscope to examine the anatomical features and the characteristics of the external hyphal elements (hyphae, rhizomorphs and cystidia) (Agerer, 1987-2012). Based on their characteristics the examined mycorrhizas were assigned to different morphotypes, and occasionally it was possible to identify the fungal partner. Thanks to PCR-based molecular techniques for fungal identification (genotyping) (Peay *et al.*, 2008) understanding of the fungal communities of truffières has increased considerably in recent years with the internal transcribed spacers (ITS) of rDNA, the PCR target region for the taxonomic identification of ECM fungi.

The comparison of the fungal ITS sequences obtained from a morphotype against those deposited in International Nucleotide Sequence Database (INSD: GenBank, EMBL, and DDBJ) has allowed us to identify the taxa of the symbiotic fungi at genus or even species level or, at worst, to assign it to one of the main ECM lineages (Telephoroid, Sebacinoid, Agaricoid, etc.) (Zambonelli *et al.*, 2012). For example, molecular analyses have made it possible to identify the AD morphotype (Giraud, 1979), as *Trichophaea woolhopeia* (Cooke & W. Phillips) Boud. which is one of the most common ECM fungi in Italian, French and Spanish truffières (Baciarrelli Falini *et al.*, 2006; De Miguel *et al.*, 2014). It has also been possible to study and compare the fungal communities in productive and non-productive truffières (Leonardi *et al.*, 2013; Napoli *et al.*, 2010).

In general, where *T. melanosporum*, *T. aestivum*, *T. borchii* and *T. macrosporum* fruiting bodies are found their mycorrhizas are abundant (Benucci *et al.*, 2011, 2014; Iotti *et al.*, 2010; Napoli, 2010). Moreover, the presence of *T. melanosporum* reduces the fungal diversity inside the brûlè (burnt area) (Napoli *et al.*, 2010), which is the area of soil around the host plant generally devoid of vegetation where truffles are formed (Pacioni, 1991). In contrast, in natural productive *T. magnatum* truffières mycorrhizas are absent (Leonardi *et al.*, 2013) or quite rare (Bertini *et al.*, 2006; Murat *et al.*, 2005). This has made it very difficult to study this truffle in its natural environment.

Recently, methods have been developed to identify and quantify *T. magnatum* mycelium in the soil by Iotti *et al.* (2012). *Tuber magnatum* mycelium was found to form large patches in productive areas (Zampieri *et al.*, 2010), and using real time PCR, it was possible to determine its spatiotemporal dynamics (Iotti *et al.*, 2014). It was found that *T.*

magnatum mycelium is particularly abundant and widespread in spring and tends to concentrate in the productive areas during fruiting (autumn) (Iotti *et al.*, 2014). Using this technique it was possible to assess the effects of cultural practices on *T. magnatum* and suggest methods for its future cultivation (Salerni *et al.*, 2014). The same technique was also applied to detect and quantify *T. melanosporum* and *T. aestivum* mycelium in the soil (Gryndler *et al.*, 2013b; Parladè *et al.*, 2013). The growth rate of development of *T. aestivum* mycelium in the soil was established (Gryndler *et al.*, 2015), moreover the presence of *Tuber aestivum* mycelium in herbaceous plants was found inside the brûlè (Gryndler *et al.*, 2014) confirming previous results obtained by Plattner and Hall (1995) 20 years earlier for *T. melanosporum* using immunological techniques.

Interesting insights were obtained recently by metagenomics and metaproteomics, coupled with next generation sequencing, where the whole microbial community of an environmental sample is examined after the extraction of total DNA or RNA. These studies can be used not only to determine fungal diversity but also the bacterial diversity in soil, roots or ascomata and to elucidate their metabolic pathways (Benucci and Bonito, 2016; Zamperi *et al.*, 2016). That is particularly important because the presence of certain bacterial species may drive the composition of fungal communities favoring the development of some ECM species. For example, it was found that the ubiquitous bacterium, *Staphylococcus pasteurii*, completely inhibited the development of *T. borchii* and *Suillus luridus* (Schaeff.) Murrill (= *Boletus luridus*) *in vitro* but had no effect on *Hebeloma radicosum* (Bull.) Ricken (Barbieri *et al.*, 2005b), which as with other *Hebeloma* spp., is considered a competitor of *T. borchii* in the field (Zambonelli and Iotti, 2001).

An extensive bacterial and fungal diversity has been found inside truffle ascomata (Barbieri *et al.*, 2005a; Barbieri *et al.*, 2007; Benucci and Bonito, 2016; Gryndler *et al.*, 2013a; Pacioni *et al.*, 2007). Some of these can rot truffles (Figure 2a, b, c and d) and can result in significant losses particularly in Australian truffières (Eslick, 2012, 2013). Some of the bacterial pathogens have defied cultivation which has limited our understanding of how they might be controlled. Other uncultivable bacteria seem to be selected from the soil communities during the early stage of truffle formation and may be beneficial to truffle growth. The genus *Bradyrhizobium* seems to be specific to the ascomata of *Tuber* spp. (Antony-Babu *et al.*, 2014; Barbieri *et al.*, 2005a; Barbieri *et al.*, 2007; Benucci and Bonito, 2016; Gryndler *et al.*, 2013a). Bacteria closely related to *Bradyrhizobium* spp. and bacteria belonging to Epsilonproteobacteria and Firmicutes subdivisions may be able to fix nitrogen inside *T. magnatum* ascomata, and perhaps could have a key role in fruiting body nutrition (Barbieri *et*

al., 2010). Interestingly, the β -Proteobacteria found in *T. borchii* fruiting bodies could produce thiophene volatiles which are major contributors to the human-sensed aroma of *T. borchii* (Splivallo and Ebeler, 2015) by biotransformation of nonvolatile precursor(s) into volatile compounds (Splivallo *et al.*, 2015).

Recently viruses have also been found inside *Tuber excavatum* Vittad. and *T. aestivum* (Stielow and Menzel, 2010; Stielow *et al.*, 2011a, 2011b, 2012). Infected truffles are apparently symptomless, but it is still not clear whether the presence of mycoviruses in truffles affects fruiting body formation, mycelium growth or its ability to form mycorrhizas. Recently, a virus belonging to the genus Endornavirus was found inside *T. magnatum* fruiting bodies and reported to produce external brown spots (Ratti *et al.*, 2016). If confirmed, this species would be the first evidence of a symptomatic mycovirus in a truffle causing severe reduction of economic value (Figure 2e).

Another important ecological aspect which could have an impact on truffle cultivation is the role of animals (e.g. mammals, insects) on spore dispersal and germination. Recent studies showed that inoculating plants with *T. aestivum* spores digested by a pig, increased their ability to in-

fect *Quercus robur* L. plants under greenhouse conditions (Piattoni *et al.*, 2014). These results indicate that we should reconsider the importance of animals, such as wild boars in truffle cultivation (Salerni *et al.*, 2013).

GENETIC ADVANCES AND THE FUTURE

Over the past 20 years numerous genetic studies have been carried out on truffles. These were aimed at understanding the genetic mechanisms which regulate mycorrhizal formation (Polidori *et al.*, 2002), the formation and maturation of the fruiting bodies (Abbá *et al.*, 2007; Zeppa *et al.*, 2002), the origin and evolution of truffles (O'Donnell *et al.*, 1997; Percurdani *et al.*, 1999; see Bonito and Smith, 2016 for a review) and population genetics (Bertault *et al.*, 2001; see the book by Zambonelli *et al.*, 2016). The genome sequencing of *T. melanosporum* has opened new frontiers in the study of truffle genetics which has led to a better understanding of truffle cultivation (Martin *et al.*, 2010). For example, it has been confirmed that Minter's 1985 observation (in Hall *et al.*, 2010) that truffles have a sexual life cycle is correct and that it is also heterothallic *i.e.*, fruiting body formation requires two different strains of different mating type to meet (Paolocci *et al.*, 2006). The two idiomorphs of the mating types, MAT1-1-1 and MAT12-1, were

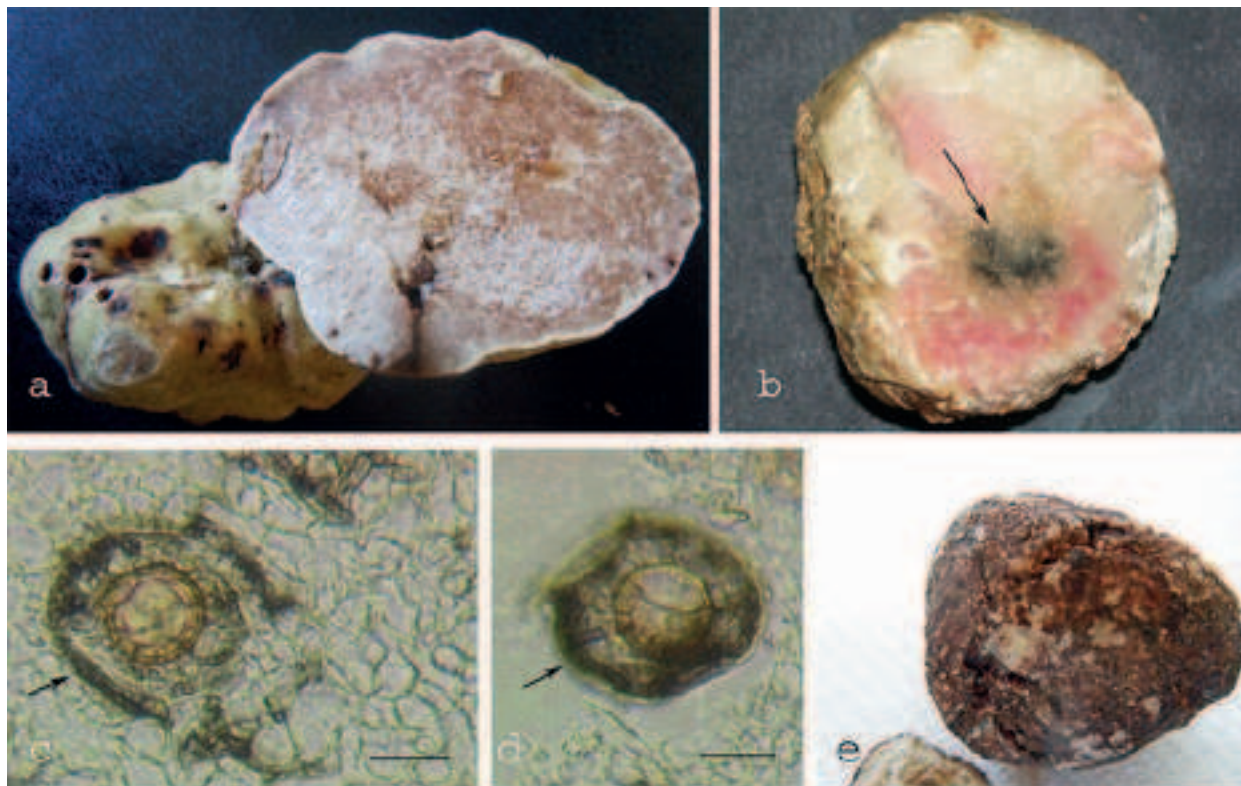


Figure 2. *Tuber magnatum* ascomata: a) healthy, b) affected by a bacterial and a central fungal infection (the arrow indicates the fungal infection), c and d) microscopic observations of the fungal infection of fig. 2b. The arrows indicate the hyphae of the invasive fungus (bars = 20 μ m), e) affected by a mycovirus showing an anomalous brown peridium.

characterized in several *Tuber* species (*T. melanosporum*, *T. indicum* and *T. borchii*) and specific primers to amplify them were designed (Belfiori *et al.*, 2013, 2016; Rubini *et al.*, 2011b).

Once we were aware that truffles had a sexual life cycle, inoculating plants for commercial use with mycelial cultures was deemed too risky. This is because pure cultures of mycelia are isolated from the gleba of fruiting bodies that is composed solely from maternal hyphae and hence only the maternal mating type. However, now that we are able to identify the mating type genes of *Tuber* mycelia, we can select strains of both mating types for inoculation purposes (Zambonelli *et al.*, 2015). Recently the first truffles were harvested from trees inoculated with mycelial cultures carrying different strains of *T. borchii* demonstrating that in the future it will be possible to use this technique on a commercial scale (Iotti *et al.*, 2016). Mycelial inoculation offers numerous advantages such as the possibility to completely exclude contamination from other *Tuber* species in the inoculum, which, as discussed in the previous paragraphs, may create competition in the field. Moreover, in the future it may be possible to select the fungal strains used based on their genetic characteristics such as suitability to a specific set of ecological conditions.

After the discovery of the heterothallic nature of truffles, studies were carried out to better understand the distribution of the mating type in natural and cultivated truffières. In Australia, where *T. melanosporum* was introduced by cultivation, the scarce productivity of some truffières was attributed to low genetic diversity or to the absence of both mating types (Linde and Selmes, 2012). The results obtained showed that both the mating types were in fact present in the truffière, even if not always on the same plant. Instead, the major problem was confirmed to be the contamination of the plants with *T. brumale* at planting time. Surprisingly, like in productive Italian and French truffières, the two mating types were distributed in different patches inside the truffières (Murat *et al.*, 2013; Rubini *et al.*, 2011a). This now poses the question as to how the two mating types meet allowing fertilization and fruiting body production. Current theory suggests that mitospores are involved in fertilization (Iotti *et al.*, 2016) after mitotic spore mats of truffle species have been described (Urban *et al.*, 2004; Healy *et al.*, 2013), but Le Tacon *et al.* (2016) warned that this issue remains to be resolved.

CONCLUSIONS

Undoubtedly the major item of expenditure on edible mycorrhizal mushroom research over the past decade in Europe has been the sequencing of the *Tuber* genome, the discovery of the heterothallic nature of *Tuber*, the existen-

ce of mating types and idiomorphs characterized, and their distribution in natural and cultivated truffières. From this, it has been demonstrated that mycelial strains carrying the mating types can be used to inoculate plants and produce truffles opening the use of this technique for the production of mycorrhized plants for truffle cultivation.

Advances in soil molecular ecology have given important insights into truffle biology and ecology offering a better understanding of the interrelationships between truffles and associated soil organisms. Some microbial groups could have a crucial role in mycorrhizal formation and the maturation of fruiting bodies suggesting possible ways microbiota might be exploited to improve truffle cultivation.

There remain huge gaps in our understanding of truffle biology. For example, it is still not known how or where the mating strains get together, and whether mitospores are involved in this process. The roles of bacteria in truffle ecology is still not clear either. The lack of funds for basic truffle research and studies to improve truffle yields is a major barrier to bridging this gap. For example, in the 1970s and 1980s various organic mixes were touted as truffle stimulants, whereas they reduced the number and weight of truffles from producing trees. Other products are now also being applied in truffle producing countries with little or no statistical data to back up claims that they stimulate truffle production. Indeed, in some instances productive truffières have ceased producing truffles following the application of such "fertilizers". In the absence of specialists investigating these new products it is left for the market or consumer rights legislation to decide the efficacy or otherwise of such products.

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