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Review

Status and prospects of research on edible fungi protoplast fusion technology

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Abstract
Protoplast fusion technology originated from cell-cell fusion technology in the 1950s and was initially applied to animal cells. The advantage of applying it in the field of edible fungi breeding is that compared with conventional natural selection and hybrid breeding methods, it could more effectively overcome certain incompatibility obstacles to achieve distant hybridization, and more purposefully select parents to obtain new varieties with outstanding characteristics. For these reasons protoplast fusion technology is becoming of great significance for the improvement of edible fungi strains, the breeding of new varieties, and the taming of difficult-to-cultivate species. At present, relatively stable and capable-of-fruiting fusants are limited to several genus and species, and using protoplast fusion for distant hybridization requires new breakthroughs overcoming vegetative incompatibilities. Technically, the acquisition and identification of fusants could also be improved to a more controllable approach. This article focuses on the longitudinal discussion of the role of protoplast fusion technology in the genetic breeding of edible fungi. It explains: i) the preparation and regeneration of protoplasts in the process, ii) the specific methods of fusion, iii) the identification, mutagenesis, and rejuvenation of fusants, and iv) possible impacts of two incompatible systems. Certain application practice cases are also introduced to summarize its promotion prospects.

Keywords
protoplast, fusion, edible fungus, mutagenesis, vegetative incompatibility

Introduction

Protoplast is a morphological unit, which contains all organelles, some cytoplasm, one or more nuclei and only the membrane after removing the cell wall (Hocart and Peberdy, 1990). As no cell wall to bind the shape, protoplasts are also called spheroplasts (when in bacteria the cell wall is lacking but both outer and plasma membranes are present) for their standard spheres (Nishida, 2020). Animal cells themselves are equivalent to protoplasts and cell fusion simply take place by the breakdown of two juxtaposed membranes then the reshuffling of cytoplasm, nuclei, and organelles of both cells into a single, viable one (Shinn-Thomas and Mohler, 2011). However, in cell engineering of plants,

bacteria, and fungal somatic cells, the first and most important step of protoplast fusion is to remove the sturdy cell wall, for which the commonly used method is enzymatic breaking (Cocking, 1972; Peberdy, 1980; Alföldi, 1982). In 1960, cell biologists first used Sendai (or HVJ/Sev) virus inducing method to intentionally fuse isolated mouse cells (Okada, 1958; Ogle and Platt, 2004). Then protoplast fusion technology was applied to fungi (De Vries and Wessels, 1972) and plants (Kao and Michayluk, 1974) a dozen years after. Since Yoo et al. (1987, 1989) used protoplasm fusants for *Pleurotus* breeding, protoplast fusion technologies started to develop rapidly in the edible fungi industry.

Although the fields of genetic engineering and gene editing have made strides of progress, the application of protoplast fusion technology in biological breeding and germplasm innovation still have irreplaceable advantages (Pelletier, 1993; Zhang et al., 2023). Crossbreeding of edible fungi is usually restricted due to the presence of cell walls, and the existence of incompatibility systems is an obstacle to fusant acquisition, development, and fruiting (Singh and Kamal, 2017). Thus, protoplast fusion technology applied in edible fungi breeding must be continuously tailored to the species under consideration. Given that in Asia, the demand for edible fungi is rather huge, allowing the research of germplasm resources gain great market potential, the authors here review the progress of protoplast fusion technology in edible fungi, starting from the preparation, regeneration, fusion, mutagenesis, rejuvenation, application and vegetative incompatibility, and with a prospect of providing guidance for further exploring its potential development. Certain information in this review is from articles written in Asiatic languages which is here summarized and reported for a wider scientific audience.

Preparation and regeneration of protoplasts

Selection of parents

The selection of starting strains is important because the complementary advantages (e.g., excellent traits as greater growth rate and metabolism, or improved disease/insect/stress resistance, vitality, and mating ability) and high productivity of both parents need to be considered in both scientific research and the production process (Sun et al., 2002). In addition, selecting parents aims also at identifying fusants after fusion of marked strains. If they are marked, then regenerants of both parental strains can be excluded when selecting fusants, omitting part or all the identification work (Chen et al., 2013):

a) Resistance marker strains. In 1984, Bradshaw and Peberdy (1984) first used this marking method in the fusion of *Aspergillus nidulans* (Eidam) G. Winter and *A. rugulosus* Thom & Raper. The target fusant was selected from the products. If there are no natural resistance markers, resistance can also be acquired through various mutagenesis methods (Bradshaw and Peberdy, 1984). Cao et al. (1998a,b) have also reported that protoplast fusion between cycloheximide-resistant and carbendazim-resistant *Ganoderma* parent strains, generates fusants with dual resistances; the selecting of protoplast fusion resistance markers of parent strains can provide a basis for target fusant isolation (Cao et al., 1998c);

b) Inactivated strains. Unlike simple resistance filtering, one or both parent strains are inactivated before fusion by chemical agents such as iodoacetamide (CIA) or physical treatments as heating, ultra-violet or rays (X or γ, the γ always applied in asymmetric fusion technique) (Guo et al., 1993). After using appropriate methods to treat the protoplasts of a single parent (or both), the physiological structure of a small part of these protoplasts should be damaged and lose activity and the ability to regenerate. Damage sites caused by different inactivation methods are different, but

metabolic complementation of the "lethal" injury site can occur after fusion and regenerative fusants could be obtained under specific conditions (Böttcher et al., 1989; Liang et al., 1999). Protoplast fusion with inactivation of a single parental protoplast could be taken as a unidirectional transmission of genetic information. When both parental protoplasts are to be considered for inactivation before fusion, however, based on the principle of damage complementation (Wright, 1984), the treatment against each parent must be different (Fodor et al., 1976; Li and Chen, 1984; Luo and Li, 1989), so the inactivated protoplast may be the cytoplasmic donor/recipient or the nuclear donor/recipient of the fusant. For example, radiation inactivation preferentially eliminates nuclei, so irradiated parental protoplasts are suitable cytoplasmic donors (Sidorov et al., 1981). Heat inactivation causes many functional enzymes and proteins in the cytoplasm to lose their original activity (Kucsera et al., 1998; Xin et al., 2020). A practical case for this is that after the protoplasts of *Lentinus squarrosulus* Mont. (syn. *Lentinus subnudus* Berk.) (resistant to a temperature of 35 °C) were inactivated at 40 °C and then fused with the not inactivated protoplasts of *L. edodes* (Berk.) Pegler (not resistant to high temperatures), the fusants of the two parents could be directly screened out under 35 °C culture conditions (Peng and Lu, 1987). In addition to CIA, radiation or heating, inactivation could also be achieved by using antibiotics, ultraviolets or other biochemical agents (Tang et al., 2010), as long as attentions on the inactivation treatment time, temperature, concentration or intensity, etc. are carefully paid (He et al., 2020);

c) Auxotrophic strains, which lack one or more enzymes necessary to synthesize specific organic compounds, must be supplied in the media to allow strain growth. If the parents are auxotrophic, the enzyme system should be complementary after fusion and fusants could be identified using minimal culture media (Zhang, 2003);

d) Fluorescent-marked strains. After staining parental protoplasts with different fluorescent dyes, the fusants with different fluorescent colors can be selected under a fluorescence microscope (Cheng et al., 1997; Yan et al., 1997; Li et al., 2002a);

e) Molecularly characterized strains. The molecular characteristics of parents also serve as a reference for fusant identification. See also in *Identification of the fusants*.

When selecting the starting strain, the characteristics of the hyphae are crucial. Usually, it would be more easily to success preparing protoplasts from activated, young and vigorous parents, because releasing protoplasts is difficult for aging or degenerated hyphae (unless the purpose is to rescue aging or degenerated strains) (Davis, 2020). It is best to use monokaryotic hyphae, because dikaryotic or multinucleated hyphae are not conducive to the expression of fusion traits and the stability of the fusion (Ogawa, 2018).

The activation, regeneration of parents and the osmotic stabilizer

The commonly used parental materials for protoplast isolation from edible fungi are mycelium and basidiospores (Aswini et al., 2014). Different mycelium culture methods also have different impacts on the preparation of protoplasts. For example, the protoplasts of *Morchella conica* Krombh. formed from mycelium activated by solid medium are less stable than those activated from liquid culture (Zhang et al., 1989). Protoplasts usually form at natural/induced breaks of hyphal tip or transverse septa, or at the germination holes of the basidiospores (Dube, 2013). The age of mycelium is always a direct factor affecting the yield, stability, and regeneration ability of protoplasts (Kawasumi et al., 1987). Walls of young mycelium are thinner and easier to be decomposed by enzymes. It is also easier to obtain protoplasts from mycelium rather than from basidiospores (Liao et al., 1987), but protoplasts

obtained from the latter are more uniform in shape, size while better for physiological and biochemical characteristics (Singh and Kamal, 2017).

The regeneration of protoplasts is a prerequisite for the regeneration of fusants. The protoplasts with the cell wall removed are extremely sensitive, and osmotic stabilizers can maintain the balance of osmotic pressure inside and outside the cell (Peberdy, 1980), preventing the protoplasts from rupture or shrinkage, and maintaining their biological activity stable until regeneration. The mechanism may be that osmotic stabilizers have a certain impact on the activity of cell wall dissolving enzymes (Li and Li, 1999), and they are known to all for their necessities on greater release rate of protoplasts, fusion rate, and regeneration rate. Protoplast regeneration is the process of restoring protoplasts to cells that can divide normally (Zhang et al., 1998). The osmotic pressure stabilizers required for the regeneration of edible fungus protoplasts could be mainly divided into two categories: organic and inorganic. Commonly used organic osmotic stabilizers include mannitol, sorbitol, sucrose and glucose, etc. Commonly used inorganic stabilizers include KCl, NaCl and $MgSO_4$. They have different effects on different edible fungus species, but the concentrations are often between 0.4-1.2 $mol \cdot L^{-1}$ (the most used is a concentration around 0.6 $mol \cdot L^{-1}$) (Lijima and Ochiai Yanagi, 1986). Many studies have also shown that inorganic osmotic stabilizers are more effective than organic ones in the preparation process. The reason may be that inorganic osmotic stabilizers can promote full contact between the enzyme solution and the cell wall and increase enzyme activity. In contrast, organic stabilizers can provide better protection for protoplasts and are beneficial to regeneration (Meikle et al., 1988). Protoplast regeneration rates of edible fungi parents are usually low, less than 10% (less than 1% in many cases) but that of some species can reach 50% (Ochiai Yanagi et al., 1985), which is mainly due to intrinsic factors or regeneration culture conditions. Researchers have also done more in-depth research on Ascomycetes such as *Morchella* spp., for whom it is more difficult to obtain protoplasts. Liu et al. (2008) found that the production yield of *Morchella crassipes* (Vent.) Pers. protoplasts could reach 2.5×10^4 individuals/mg, and the regeneration rate was 0.72%. Chen et al. (2007) found that the highest protoplast yields of *M. conica* and *M. crassipes* could reach 3.8×10^6 individuals/100 mg and 3.6×10^6 individuals/100 mg respectively, while the regeneration rates were 0.78% and 0.8%.

Selection of enzymatic solution and fusant regeneration medium

The main components of the cell wall of edible fungi are chitin and the alkali-soluble glucans (Peberdy, 1990; Bowman and Free, 2006; Novak and Vetvicka, 2008). Since the most abundant β-1,3-glucan in wall alkali-soluble glucans is bound by β-1,3-linkages (Ruiz-Herrera and Ortiz-Castellanos, 2019), walls of edible fungi are easy to be removed by alkaline extraction solution. Theoretically, chitinase and β-1,3- or β-1,6-glucanases could be priorly considered in an enzymatic system, but cell wall components of different fungal groups still differ, leading to variations in the effectiveness of lytic enzymes chosen. Cellulases were often used in the development history of the protoplast fusion technology for preparing protoplasts (Hamlyn et al., 1981; Savitha et al., 2010; Raman et al., 2021). Since cellulose is not a structural component of the edible fungus cell wall (Bowman and Free, 2006), decades of research showed that the effect of a single cellulase in hydrolyzing the cell wall of edible fungi was unsatisfactory, while the effect of a mixture of multiple enzymes more effective (Ochiai Yanagi et al., 1985). Therefore, researchers currently prefer to use a mixture of enzymatic hydrolysis agents, such as snailase. Eddy and Williamson (1957) first used snailase to dissolve the yeast cell wall and isolate protoplasts. Emerson and Emerson (1958) then

used snailase and hemi-cellulase to obtain protoplasts of filamentous fungi, thereby gradually establishing an experimental system for obtaining protoplasts through snailase enzymatic hydrolysis. In recent years, similarly, multi-component mixed enzymes with better effects include Novozym 234 (Sigma, USA) (Yoo, 2018) and Lywallzyme (Guangdong Institute of Microbiology, China).

The regeneration of fungal protoplasts is the same for both parents and fusants and involves two processes: regenerating the cell wall and returning the regenerated wall cells to their original hyphae state through cell division (Peberdy, 1995). There are three regeneration methods for protoplasts from hyphae: 1) the protoplasts produce a malformed germinative tube similar to that formed by budding cells, and normal hyphae grow at the top (*Aspergillus nidulans* (Eidam) G. Winter, *Geotrichum candidum* Link, *Penicillium chrysogenum* Thom) (Peberdy and Gibson, 1971; Dooijewaard-Kloosterziel et al., 1973; Anné et al., 1974); 2) the protoplasts first form a cell wall and maintain it (the round cells are called primary cells, from which germination tubes are produced) (Peberdy, 1979); 3) protoplasts prepared from *Boletus edulis* Bull. hyphae show a special regeneration way, that is: the protoplasts first expand and deform into thick rod-shaped “thick hyphae”, and then differentiate into "thin mycelium"(normal size hyphae) at the top (Zhang and Zheng, 1990). For the 2) model, the formation of the upper wall of the protoplast is the result of self-aggregation of macromolecular polymers that carry structurally determined information. This cell wall often lacks the mechanical properties of normal hyphal cell walls, and protoplast regeneration requires a certain amount of cell walls as a primer for regeneration, so enzymatic digestion time and fusion time need to be strictly controlled (Nečas and Svoboda, 2020). The regeneration effect of the fusion is better if sterilized mycelium extract of the strain or cellobiose and other disaccharides are added to the regeneration medium after fusion (Lijima and Ochiai Yanagi, 1986). The regeneration medium after fusion also needs a certain osmotic pressure, otherwise the fusant would be broken (Kiguchi and Ochiai Yanagi, 1985). In short, the regeneration of protoplasts and their fusants would be affected by the enzymatic hydrolysis time, the type and concentration of the osmotic stabilizer, the components of the regeneration culture medium, the culture time (age), the culture method, the ratio of the enzymatic hydrolysate, the enzymatic hydrolysis time, conditions such as temperature, pH value, etc. Multiple influences as above are always directly related to the successful breeding of new varieties (Toyomasu and Mori, 1987).

Fusion of protoplasts

Protoplast fusion technology could, at a certain level, overcome the problem of reproductive isolation due to distant genetic relationships under traditional crossbreeding methods, which makes hybridization impossible. Theoretically, protoplasts of any species or variety can be fused, but only to the physical extent. The key to a successful fusion, within a certain range, lies in gene exchange, and whether nuclear fusion can occur between parental cell nuclei. The genetic exchange is determined by the nature of the species and varieties themselves. Spontaneous fusion of protoplasts has been seen but is particularly rare, thus the scientific fusion of protoplasts always requires fusion assisting. At present, for the mainstream edible fungi protoplast fusion in the world, the fusion promotion methods are mainly divided into physical, biological and chemical.

Protoplast fusion assisting techniques

The biological assisted fusion method of protoplasm originated from the invention of the Sendai virus method. Okada (1958, 1988) accidentally discovered the phenomenon of cell fusion into multinucleation caused by Sendai virus (HVJ/Sev) in 1950s. This phenomenon occurred due to the presence of two glycoproteins, HANA protein and F protein, on the surface of the plasma membrane of the RNA virus Sendai, which have agglutination ability/neuraminidase activity and plasma membrane fusion ability respectively (Hosaka and Koshi, 1968). Therefore, the HANA protein allows the virus to adsorb to the cell membrane surface (by the agglutination ability) and then hydrolyze the cell membrane surface glycoprotein (by the neuraminidase activity). The F protein, which is composed of two subunits F_1 and F_2 (connected by disulfide bonds), could then produce two subunit fragments (F; and F:) by proteolytic cleavage of a precursor (F_0), and the fusion activity of the F protein is activated (Okada, 1993). Harris and Watkins (1965) did massive work and proved that such viruses can still be functional after inactivation by ultraviolet light or breaking by ultrasound, because the effective fusion-inducing factors (receptors) lies in the membrane of the target cells (Hernandez et al., 1996). Saga and Kaneda (2015) then further confirmed that after adjusting the HANA expression of Sev, the toxicity of viral materials to cell fusion and transfection could be significantly reduced. Currently, this virus method is widely used to study the transfection of various molecules including plasmid DNA, siRNA, proteins, and antisense oligonucleotides (Nakanishi et al., 1998; Saga et al., 2008). It can induce fusion between cells of different species of eukaryotes and has since then often been used in animal hybridization. The main problem of biological approaches lies in high cytotoxicity that may affect early-stage development of fusants, as well as complex operations in the virus preparation process, large differences in potency of inactivated biological materials, poor repeatability and low fusion rates, etc. Therefore, it is now mainly used for laboratory research instead of production.

The earliest chemical protoplast fusion method was the sodium-induced one (Verma et al., 2008). After the 1970s, chemical fusion agents could be mainly divided into five categories: a) higher fatty acid derivatives; b) liposomes; c) divalent cations; d) water-soluble polymer compounds with special structures; f) water-soluble proteins and polypeptides (Garcia et al., 1984; Bravo and Evans, 1985). The sodium-induction method causes less damage to protoplasts, but the problem is that the fusion rate is particularly low and the effect on cells with advanced vacuolation is extremely poor (Gad, 1983). Keller and Melchers (1973) discovered that high Ca^{2+} and high pH could induce fusion. Melchers and Labib (1974) used this method to successfully induce fusion of two light-sensitive mutants in tobacco species and obtained more than 100 somatic hybrids. Kao and Michayluk (1974) first used polyethylene glycol (PEG) as a fusion agent to induce the fusion of soybeans and barley, soybeans and corn, vetch and peas. In the same year, Bonnett and Eriksson (1974) discovered that diluted PEG can cause extensive cell fusion as a coagulation factor for plant cells. Pontecorvo (1975) successfully used PEG for animal cell fusion in 1975. This chemical fusion method is simpler to operate than the Sev method. Within the effective concentration range of PEG (40%-50%) (Köhler, 1980), it is also less toxic to cells than the virus. PEG is highly hydrophilic, and its role in promoting melting is not only to promote cell aggregation, but more importantly, to cause changes in the properties of cell membrane proteins and lipids and aqueous solutions in the fusion system. Tilcock and Fisher (1982) found that when the PEG concentration reached 38%, hydrogen bonds between PEG and H_2O molecule could be formed, and the free H_2O in solution disappears. Increasing the PEG

concentration can reduce the number of water molecules bound to phospholipids, leading to cell dehydration and changes in membrane structure to promote membrane fusion. Changes in the polarity of molecules around the hydrophilic medium also affect the structure of cell membrane proteins, which is conducive to the occurrence of interaction and fusion between the lipids of the two cell membranes. A slight alkaline environment around pH 8.0 is more conducive to induction (Sharon et al., 1980). After fusion occurs, the induction reaction can often be terminated by diluting the PEG concentration (Shoenfeld et al., 1982). Other chemical methods that can induce cell fusion are not as effective as the PEG one, especially in agricultural products and food products, where PEG is a broader-spectrum inducer. Therefore, since 1975, the PEG chemically induced fusion has gradually developed into a standardized chemical method. However, since a) the toxicity of PEG to cells cannot be ignored, b) the fusion rate after PEG induction is still limited; c) the cell fusion process cannot be directly observed during the process, PEG is still not an ideal assistant. Thus, in the early 1980s, electrical fusion technology emerged as the times required.

The principle of physical fusion methods is always to use physical means such as electric fields, lasers, ultrasound, etc. to fuse protoplasts. The advantages are controllable conditions, high fusion rate, non-toxicity, easy operation, and less contamination of the protoplasts suspension obtained. However, the rather high equipment requirements cause the physical fusion method basically not considered in current protocols. As early as 1974, protoplast fusion of *G. candidum* had been achieved through simple centrifugation (Ferenczy et al., 1974), but most of the protoplasts were damaged during the process. The cell membrane of plant protoplasts will also be reversibly broken down under the action of a short-term strong electric field (Yamada et al., 1979). In 1980, Neumann et al. used high-voltage pulses to successfully fuse a large number of eukaryotic microbial cells. Then after the successful fusion of animal somatic cells (Zimmermann, 1982), the physically induced fusion method by electric fields has been applied to artificial membrane systems, changing the past random cell fusion model and making it more efficient and controllable. Under the action of electric fields and high-intensity instantaneous electrical pulses, protoplasts will lose their low permeability characteristics within a few minutes and then recover. Thus, when the electric breakdown occurs in the contact area of two protoplasts, it can promote their fusion (Zimmermann et al., 1978; Zimmermann and Vienken, 1982; Zimmermann et al., 1985). Only by conducting electrofusion in low conductivity solutions, such as mannitol, sucrose, glucose and other non-electrolyte solutions, can the death of fusants due to the heat generated by the alternating current be avoided. The mutual contact between the two protoplasts during electrofusion, however, can be achieved through special fusion chamber, filtration, centrifugation, and wall adhesion (Ušaj et al., 2010). Electrofusion is a cell fusion induced in a controlled manner with spatial orientation and time synchronization. It has a high fusion rate, no cytotoxicity, is simple to operate, and can directly observe under a microscope. It currently shows great advantages in the field of medical research (Yu et al., 2008). However, the disadvantages of electrofusion are that it is equipment-dependent, different cells have different breakdown voltages, pulse buffers, and different recovery conditions after electrical treatment. Though with higher fusion rate, the low survival rate of cells after fusion is still the fundamental problem.

In summary, though cell fusion has been around for a long time, various technologies inevitably have limitations. The current progress of emerging assisted fusion methods is mainly based on physical techniques: chip-assisted fusion technologies (Yang et al., 2010), spatial cell fusion

technology (Dudin et al., 2016), ion beam cell fusion technology (Yu, 2007) and asymmetric cell fusion technology (Yamashita et al., 1989). Among which, chip-assisted (chip: a micro-cavity/ discrete microelectrode structure) could mainly be divided into microfluidic chip-based and high-throughput chip-based (Acharya et al., 2009). These emerging fusion technologies have not yet become mainstream in edible fungi, but each has its development prospects. Ion beam fusion technology can realize the ultrastructural processing of cells before fusion (cutting chromosome fragments, eliminating some chromosomes or their fragments) (Yu, 2007). Spatial fusion technology promotes cell fusion by immobilizing fusion focus through spatial focalization (Dudin et al., 2016). The microfluidic system can be used to perform correct cell pairing before fusing cells, which is to say, to reduce multi-cell fusion and to avoid fusants within a single parent (Skelley et al., 2009).

Post-fusion and regeneration

Identification of the fusants

Common biological, chemical, or physical fusion methods could not control the parents of the fusion like microfluidic chips do. To avoid products of the fusion of single parent with itself, biological identification methods are usually required:

a) Colony phenotype test: the normal regenerated colonies of the fusants should be different from the parents in terms of mycelium thickness, growth rate, colony morphology, and matrix pigment secretion, often showing traits in between the two parents, which could be morphologically observed and compared (Peberdy, 1980). For auxotrophic parents, the ideal fusant should no longer have the corresponding phenotype due to complementation of the parents (Kiguchi and Ochiai Yanagi, 1985).

b) Antagonism test: it is related to vegetative incompatibility. If the fusant is different and incompatible from the two parents, a confrontation zone or an antagonism line ("barrage") should be formed between the fusant and the parent colony (Esser and Meinhardt, 1984; Leslie, 1993);

c) Nuclear phase observation: the regenerated hyphae of the protoplast fusion are firstly characterized by differences in the nuclei from the parents (Vazquez et al., 1997). Hyphae of the parent and the fusants could be compared microscopically after nuclear fluorescence staining. Especially in the early stages of fusant germination, the nuclear distribution and nuclear behavior could more intuitively prove whether the fusion was successful.

d) Fruiting test: successful fusants are hybrid heterokaryons, and they may or may not produce fruiting bodies as edible fungi. If fruiting bodies can be produced in artificial culture, the traits of the fruiting body should usually be between those of the two parents, or, it carries the characteristic traits or markers of both parents (Li et al., 1992; Wang et al., 2003). However, the current problem is that most research on protoplast technology of difficult-to-cultivate edible fungi remains in the preparation and regeneration process (Moriguchi and Kotegawa, 1985).

The biochemical identification methods, however, mainly involve comparative identification of the fusant and its parents at the level of proteins and enzymes. The commonly used ones are mainly:

a) Soluble protein gel electrophoresis pattern analysis: this is an effective method for classifying microorganisms and a reliable method for detecting the degree of similarity between species (Carlson and Vidaver, 1982). Therefore, it can be used as a basis for fusant identification (Aggag et al., 2017), and the changes in protein content during hybrid formation need to be visually followed.

b) Isozyme electrophoresis pattern analysis: differences in the molecular structure of isozymes cause different electrophoresis bands to be formed between different species of edible fungi and even between different strains (Wang et al., 2003; Zhang et al., 2005a). Common ones include esterase isoenzyme (Pupilli et al., 1991; Kim et al., 1997; Dhitaphichit and Pornsuriya, 2005), peroxidase isoenzyme (Pupilli et al., 1991; Kim et al., 1997), SOD isoenzyme, etc. (Chakraborty and Sikdar, 2008). Among them, the esterase isoenzyme is particularly stable in strains and considered as the best choice.

Finally, modern molecular biology techniques facilitate the identification of fusants at the molecular level. The molecular biology method compares the fusion and the parent strain at the genetic level. It is more scientific, accurate and convincing than other identification methods. Decades ago, the commonly used ones include PCR amplification of important DNA segments (Khush et al., 1992) and methods using random markers such as: RAPD (Random Amplified Polymorphic DNA) (Chen et al., 1998; Luo, 2001), AFLPs (Amplified Fragment Length Polymorphisms) or RFLPs (Restriction-Fragment Length Polymorphisms) (Gu et al., 2003; Li, 2001), and pulse electrophoresis technology-protoplasts karyotype analysis (Larraya et al., 1999). These methods are still practical, but more recent accurate and labor-saving methods have been developing based on more specific molecular markers like ISSR (Inter Simple Sequence Repeat) (Lakhani et al., 2016), or primers designed on species-specific heterozygous molecular sequences (Hirpara and Gajera, 2018), or on ITS sequences (Patil et al., 2015).

Protoplast mutagenesis

Mukherjee and Sengupta (1986) used protoplast mutation against *Volvariella volvacea* (Bull.) Singer to select high-yielding varieties, which was the first case of protoplast mutation breeding of filamentous fungi. Liu et al. (1999) performed UV mutagenesis on the prepared *M. conica* protoplasts. After more than 280 seconds of irradiation, all the *M. conica* protoplasts died. After 60 seconds of irradiation, however, the selected regenerated mutant strains had been improved with not only higher biomass but also greater amino acid content (Liu et al., 1999). Regardless of whether the fusion would be done, mutation breeding of all kinds was generally carried out for parental protoplasts (Jin et al., 2000; Li et al., 2002b; Yang et al., 2002; Li and Li, 2006; Zhang et al., 2005b; Wang et al., 2006) to achieve genetic improvement and better germplasm. Protoplasts are ideal mutagenic materials because edible fungus hyphae are multicellular and not suitable as mutagenic materials. Though basidiospores are single-celled and mostly single-nucleated, their wall thickness makes it difficult for mutagens to penetrate. The protoplasts with the cell wall removed or fusants are very sensitive to the external environment and can easily mutate after being treated with mutagens.

In protoplast mutagenesis, there are two main categories of mutagens: physical mutagens and chemical mutagens. Judging from previous studies, physical mutagens are more often used, and most of whom have chosen ultraviolet rays or γ rays (UV is also often used to mark parental protoplasts for inactivation purpose). The more popular chemical mutagens were nitrosoguanidine, ethyl methanesulfonate, diethyl sulfate, and nitrosylmethylurea. The function of chemical mutagens is to alkylate the bases, causing the exchange of G:C and A:T, thereby causing the conversion or transversion of base pairing during DNA replication (Shrivastav and Essigmann, 2010).

Pre-fusion mutagenesis thus is always preferred than the post-fusion mutagenesis (Talbot et al., 1988; Stasz, 1990; Hatvani et al., 2006) because the mutations in parental protoplasts also need to be recorded and selected. The combination of protoplast fusion and mutagenesis has the potential to

increase production and accelerate breeding progress (Li et al., 2023; Qian et al., 2023). For example, if two parental protoplasts are subjected to several mutagenesis treatments separately (Lu et al., 2002; Han et al., 2004) and fused to fully exchange the genetic materials between each other, it might be possible to screen out the fusants with advantages accumulated from each mutagenesis that can effectively improve the target traits.

Protoplast rejuvenation technologies

Strains/spawn developed from successful fusion may also face the problem of direct death, aging or degeneration, thus require rejuvenation technologies. The fusant itself needs rejuvenation just after establishment. Since the protoplasts are in an osmotic stable environment from preparation until fusion while the upper wall of the fusants often lacks the mechanical properties of the normal hyphal cell wall, they are prone to rupture or their growth might be blocked in hypotonic solutions or media (Mishra et al., 2022), thus isotonic rejuvenation for fusants is required (Arnold, 2018). Meanwhile, as mentioned earlier, any fungal protoplast that originally contained an intact nucleus has the repair potential to revert to a cell with complete structure and function (Davey et al., 2005). However, some fusants do not achieve their totipotency (Nečas and Svoboda, 2020). There are two factors that can affect the development of regenerative cells. One is the physiological state of the fusant, including whether it is a vegetive incompatible heterokaryon facing programmed cell death (see "*vegetative incompatibility and protoplast fusion*"). It is also worth noting here that when protoplasts are isolated, some organelles and even nuclei are often lost (Magae et al., 1985). The other factor affecting regeneration is the external condition of culturing, including osmotic stabilizer concentration in culture medium. Only by breaking through the limitations of physiological status and optimizing external conditions can we achieve better rejuvenation.

New strains/spawns developed from the successful fusant may also be prone to aging or degradation. Generally, as consequences of fungal strain aging or degeneration, the cell vitality declines, pigment secretion increases (He et al., 2019), vacuoles increase and are easily ruptured (Liu et al., 2003). The mycelial growth becomes slow, the ability to resist contamination becomes weak, and the enzymes production decreases (He et al., 2019; Zhao et al., 2023), as in case of the Ascomycetes *Saccharomyces cerevisiae* (Desm.) Meyen, *Neurospora* spp., *Morchella* spp., and *Podospora anserina* (Rabenh.) Niessl (He et al., 2015; Osiewacz and Bernhardt, 2013). It is necessary to point out that the regeneration process of fusants includes the reshuffling of cytoplasm and organelles, cell wall regeneration, cell structure and function repair, cell division and germination. The later three are interrelated but independent physiological processes (Guo and Yang, 2010). Therefore, the protoplast fusion technology itself has long been an approach to help overcome aging and degradation to maintain on the fusants excellent traits of parental strains.

Since fungal degeneration is hereditary (Sun et al., 2017; Kour and Rath, 2016), fusant degradation may originally come from parental strains. If the selection of a degenerated parent is necessary under some special conditions, effective mutagenesis is suggested to be applied to parental protoplast (Figs 1c-d) to rejuvenate. The protoplast fusion of this parent with a healthy parent can also alleviate certain degeneration (Figs 1a-c, 1e).

Protoplast rejuvenation methods include:

a) Provide suitable environmental conditions. When the fusants are transferred from hypertonic environment to ordinary culture media, the effect of choosing a sandwich medium is always better than a single-layer medium (Sinha and Sarkar, 2023) (Figs 1a-c, 1e-g). The lower layer of the

sandwich medium is generally an osmotic stable 2.0% agar medium and the upper layer is usually a stable 0.8% soft agar medium (Wu et al., 2006), under this condition the fusant strain avoids disruption, serious aging, or death. Different culture environment conditions, including culture media condition, can stimulate the growth of fusant strains and spawns.

b) Use purification to rejuvenate. Heterogeneous individuals (subclones developed from a single fusant cell that differ in growth rate, infection ability, resistances, etc.) in the cultured mycelial population after fusion could be selectively removed (Bian and Xiong, 2009). As the consistency being improved, numerous subcultures being carried out, and inferior strains being eliminated, resistance and pathogenicity tests could then be conducted.

c) Use protoplast technology again. Protoplast and protoplast fusion technology themselves are both breeding technology and rejuvenation technology (Hospet et al., 2023). Protoplast regeneration rate generally does not exceed 2.5%, and protoplasts that can regenerate themselves are advantageous.

d) Tissue separation in robust individuals. When fruiting is possible for the fusant, continuously select those individuals with excellent traits for isolation or perform multiple rounds of fusion.

e) Isolation from mycelium tips. It is one of the best measures to judge whether the fusion is successful or fake. It can achieve rejuvenation for strain/spawn also because tip-isolation is an effective measure for virus removal (Liu et al., 2017). The only problem is that the probability of genetic mutation rate at the tip of hypha is quite high, though the direction could be positive.

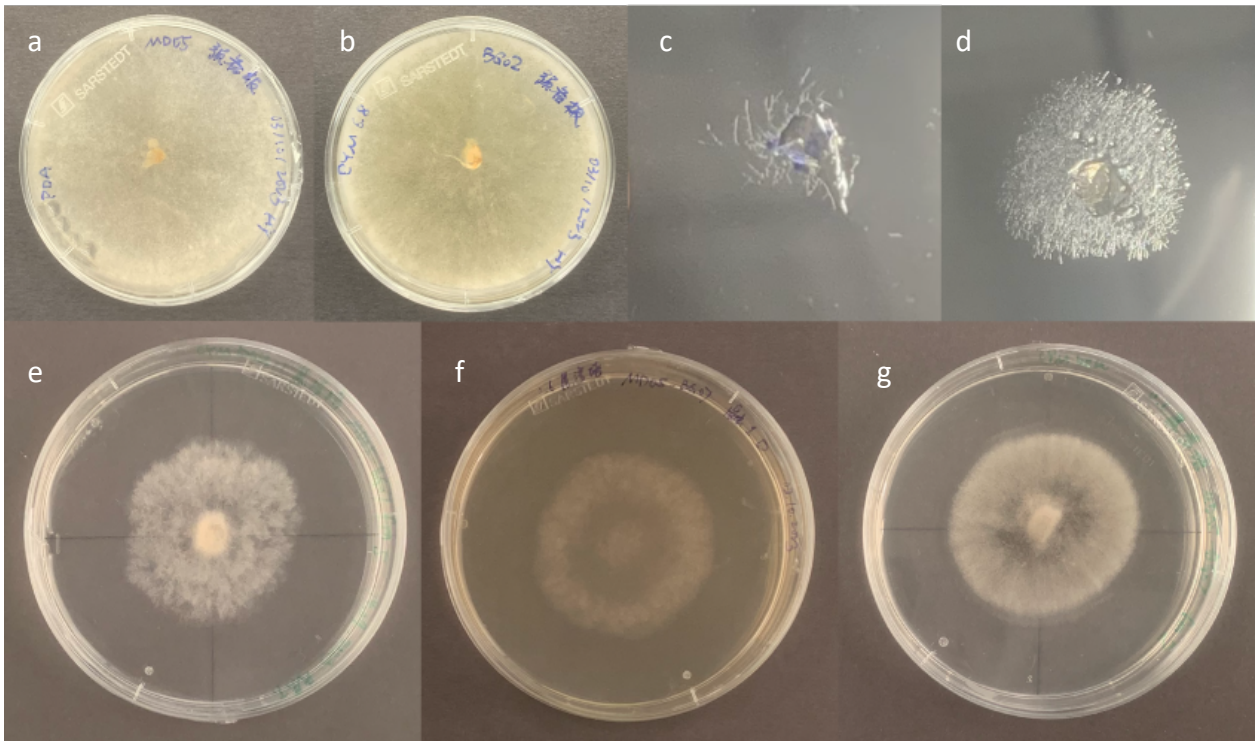

Fig. 1. – a) Healthy parental *Morchella eximia* Boud. Strain MD05 on PDA 6.8; b) Healthy parental *Morchella sextelata* M. Kuo strain BG02 on CYM; c) Degenerated parental *M. sextelata* BG07 (from single protoplast) after regeneration; d) Ultra-Violet rejuvenated parental *M. sextelata* BG07; e) Aging and degenerated strain (colony shaped like overlapping folding fans) developed from the fusant of degenerated *M. sextelata* BG07 and healthy *M. eximia* MD05 on CYM; f) Rejuvenating fusant on sandwich medium; g) Rejuvenated fusant on CYM (All pictures were taken when the *Morchella* colonies were 5 days old).

Application of edible fungi protoplast fusion in breeding

Hopwood et al. (1977) proposed that protoplast fusion may expose recessive genes through recombination, causing the expression of some recessive genes or randomly generating new gene phenotypes. However, in the past 50 years, due to the slow progress of research on the formation of fruiting bodies from distant complementary mating strains, current research results on fusion breeding of edible fungi protoplasts are still mostly limited to obtaining intraspecific, interspecific and intergeneric fusants. Previous researchers believe that the reason may be that a widely applicable fusant nuclear mitosis technology has not been developed (Tan et al., 2001).

In the 1970s, De Vries and Wessels (1972) isolated protoplasts of *Schizophyllum* sp. for the first time. In the mid-to-late 1980s, protoplast fusion technology began to be gradually applied to strain improvement or *in vitro* variety selection (Kovylyaeva et al., 2007), though most successful fusion cases to date have occurred within basidiomycetes. Yoo et al. (1987) conducted protoplasmic fusion and sexuality research between several *Pleurotus* species, and later used the PEG method to achieve interspecific fusion of auxotrophic strains of the *Pleurotus*. The resulting fusion strains were not only able to produce fruiting bodies but also had greater yields than their parents (Yoo, 1992). Ohmasa (1986) also obtained *Pleurotus ostreatus* (Jacq.) P. Kumm. intraspecific protoplast fusion. Kiguchi and Ochiai Yanagi (1985) prepared protoplasts of *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo [syn. *Coprinus macrorhizus* (Pers.) Rea] and found that fusions formed by protoplasts of the same mating type could not produce mushrooms. Sun and Zhang (2000) performed interspecific fusion of *L. edodes* and *Lentinus conatus* Berk. (syn. *Lentinus javanicus* Lév.) and obtained two fusion strains, whose growth characteristics were significantly different from those of the two parents. Liu et al. (1990) conducted a fusion between Oyster mushroom and *L. edodes* and successfully obtained the fusant. Santiago Jr (1982) tried intergeneric protoplast fusion for *V. volvacea* but did not obtain fusion cells. Singh et al. (2007) performed interspecific fusion on *Agaricus bisporus* (J.E. Lange) Imbach with *Agaricus bitorquis* (Quél.) Sacc. but did not obtain fruiting bodies.

From the 1990s to the early 20th century, in depth research began to move toward the testing and analysis of parent combinations and fusants, and protoplast fusion technology began to be commercially applied to edible fungus breeding. The intraspecific fusion commercial variety “H380” of Japanese oyster mushroom was applied for approval in 1990 (the parents were Japanese oyster mushroom and non-Japanese one), and this was the world's first practical case of protoplasmic fusion in edible mushrooms (Nikkei Biotech, 1990). Subsequently, Tan et al. (1999) from China applied for the new commercial variety *L. edodes* “Shenxiang No.8” using the fruiting bodies of cultivated and wild *L. edodes* after mononucleation of protoplasts and fusion hybridization. Until now, there are but a few cases of successful fusion between genera, few cases of successful fruiting, very few cases of successful fusion between families, and very few cases of successful variety-selection and commercialization. Among the successful cases of fusion, one was between *P. ostreatus* and *Agrocybe cylindracea* (DC.) Maire, although the fusant did not successfully produce fruiting (Eguchi, 1993). While the other was a case between *Flammulina velutipes* (Curtis) Singer and *Pleurotus sajor-caju* (Fr.) Singer in which the cross-genus fusant was particularly successful and a new commercial variety “Jinfeng 2-1” was bred and approved. It has produced significant social and economic benefits (new output value of more than 600 million Chinese yuan) (Xiao et al., 1998).

In recent years, a large number of new strains have been cultivated using protoplast fusion technology with optimized traits, improved resistance to impurities, higher total biological efficiency and greater yield as targets (Wu et al., 2020). Result with successful fruiting was mainly obtained in the fusion under genus *Lentinula* (Mallick and Sikdar, 2014), *Pleurotus* (Selvakumar et al., 2015; Das et al., 2021), *Volvariella* (He *et al.*, 2018), *Flammulin*a (Kang *et al.*, 2019), *Hypsizygus* (Sun *et al.*, 2014), *Ganoderma* (Li et al., 2023), etc., but rarely seen in the fusion of difficult-to-cultivate edible fungi, within the Ascomycetes and between distant species. *Tuber borchii* Vittad. protoplasts were shown to still have no cell wall formation after 16 days of culture with saporin containing liposomes (Poma et al., 2005). The only successful case of *Morchella sextelata* M. Kuo and *Morchella importuna* M. Kuo, O'Donnell & T.J. Volk showed the traits of the fusant fruiting bodies were between those of the two parents (He et al., 2020). Also, the only success of *Cordyceps sinensis* (Berk.) Sacc. and *Cordyceps militaris* (L.) Fr. fusion resulted in two high-yielding cordycepin strains (Qu et al., 2015). Therefore, the current protoplast fusion breeding technology for edible fungi is still in the exploratory stage, and its development prospects are still broad. Currently emerging research directions include organelle transfer and gene transformation using protoplasts as receptors. It could be seen in previous studies that the nuclei separated from the mycelium of *Agrocybe aegerita* (V. Brig.) Singer were introduced into *Pleurotus florida* Singer protoplasts to obtain transfer products (Yoo et al., 1989). Tryptophan-deficient *Schizophyllum commune* Fr. (Munoz-Rivas et al., 1986) and *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo [syn. *Coprinus cinereus* (Schaeff.) Gray] (Binninger et al., 1987) protoplasts were successfully transformed into prototrophy after being introduced into plasmid DNA containing the corresponding TRP1 sequence. Vector-mediated gene transformation has opened a very useful way for research in many fields of fungal biology, including breeding (such as the study of promoters). Through organelle transplantation, we can also explore aspects such as organelle origin, cell origin, cell genetic modification, and nucleocytoplasmic relationship.

Vegetative incompatibility and protoplast fusion

The existence of incompatibility systems sometimes hinders protoplast fusion, or hinders the survival, growth and development of fusants. Generally, there are two categories of incompatibility systems prevalent in filamentous fungi (Glass and Kaneko, 2003), one controls the sexual fusion between different organisms, and the other controls the vegetative fusion of hyphae of different organisms, and these sexual and vegetative genetic systems usually differ among fungal taxa (Coelho et al., 2017). In the basidiomycetes of edible fungi, these two systems overlap because sexual fusion occurs for somatogamy, as hyphal fusion between two compatible primary hyphae (Raudaskoski, 2015).

Vegetative (or heterokaryon) incompatibility is present in mitosporic fungi and in both homothallic and heterothallic species. It has also been widely reported in edible fungi of ascomycetes, and basidiomycetes, though basidiomycetes are not amenable models for their essential dikaryotic life cycle (Glass et al., 2000; Paoletti, 2016). Detailed description of vegetative incompatibility could notably be found on *Morchella* spp. (Liu *et al.*, 2021; Chai *et al.*, 2022), *C. cinereus* (May, 1988), *P. ostreatus* (Kay and Vilgalys, 1992) *Ganoderma lucidum* (Curtis) P. Karst., *G. tsugae* Murrill (Adaskaveg and Gilbertson, 1987) *Heterobasidion annosum* (Fr.) Bref. (Hansen et al., 1993) *Fuscoporia gilva* (Schwein.) T. Wagner & M. Fisch. [syn. *Phellinus gilvus* (Schwein.) Pat.], *Coniferiporia weirii* (Murrill) L.W. Zhou & Y.C. Dai [syn. *Phellinus weirii* (Murrill) Gilb.] (Hansen

et al., 1994; Rizzo et al., 1995), etc. Under the premise it is possible that if the fused strains are vegetative incompatible a programmed cell death response could be induced in this heterokaryotic fusant (Daskalov et al., 2017). For a subset of model ascomycetes, studies on vegetative incompatibility studied the interactions between the gene products of the so-called *het* (heterokaryon) or *vic* (nutrient incompatibility) loci (Labarère et al., 1974; Mylyk, 1975). The fusion of hyphae with different *het* alleles induces heterokaryon incompatibility, leading to hyphal separation and direct death, and showing great similarities in the phenotypes of different species (Ainsworth and Rayner, 1986; Jacobson et al., 1998; Saupe et al., 2000; Aimi et al., 2002a,b). Early genetic analyses showed that there are 7 to 12 *het* loci in different ascomycetes (Glass et al., 2000; Saupe, 2000) and there was found less than five generally multi-allelic *het* loci in basidiomycetes (Van der Nest et al., 2014). However, more recent genomic studies revealed that these systems are more complicated than it was previously supposed (Iotti et al., 2012; Chai et al., 2022). It is obvious that in ascomycetes with clearer genetic backgrounds on this, each specific species possesses multiple specific *het* loci and thus increases the number of potential incompatible genotypes when crossing between distantly related parents, and greatly increases the probability of vegetative incompatibility and fusion failure (Gonçalves and Glass, 2020).

In Ascomycota, if the two incompatibility systems are independent of each other, as of *Cryphonectria parasitica* (Murrill) M.E. Barr from Diaporthales (Cortesi and Milgroom, 1998) and the edible morels (*Morchella* spp.) from Pezizales (Du and Yang, 2021; Liu et al., 2021; Chai et al., 2022), mating-type idiomorphs do not participate in the regulation of vegetative incompatibility, thus the presence of the opposite mating type could lead to both vegetatively compatible and incompatible reactions. But for some other ascomycetes like *Neurospora crassa* Shear & B.O. Dodge, the mating type locus was proved to act as vegetative incompatibility locus (Glass et al., 1990), and vegetative coexpression of opposite mating types could be lethal (Beadle and Coonradt, 1944; Garnjobst, 1953). In this case it was found a gene called *tol* that controls the expression of mating type products during vegetative or sexual phases (Shiu and Glass, 1999). For another few ascomycetes like *Tuber melanosporum* Vittad. and *T. borchii*, the fusion between different strains seems to be related to different genetic systems which prevent hyphal fusion (Iotti et al., 2012). It is currently widely believed that protoplast fusion technology can break the genetic barriers caused by sexual incompatibility, then effectively overcome the barriers of distant hybrid incompatibility factors in some edible fungi, and thus make it much easier to break through conventional sexual or parasexual reproduction (Tan et al., 2001). It is also an efficient way to allow successful fusion between vegetative incompatible ascomycetes and where the incompatibility does not lead to cell death responses (Cha and Yoo, 1997).

Prospects

Though promoting nuclear fusion of parental cells, compatibility of fusants and mitosis of fusant nuclei seem to be major problems to be solved, protoplast fusion technology still provides new ideas for breeding or hybrid breeding of edible fungi at interspecific, intragenic and even interfamily level, as well as for the optimization of artificial cultivation of some difficult-to-cultivate species.

For some ascomycetes whose mating process is necessary for single spore isolates have usually only one mating type (Linde and Selmes, 2012; Du et al., 2017), researchers could directly fuse parents of different mating types. If incompatibility does not occur or is overcome, the cultivation

cycle of this fusant can then be greatly simplified and shortened. As for mycorrhizal fungi like truffle, we could use directly the strains developed from successful fusants possessing both mating types to inoculate the hosts.

In addition, due to their own protoplast characteristics, protoplast fusants are easier to combine with various mutagenesis techniques for breeding, thereby, new strains with faster growth, lower nutritional requirements, better fruiting, and stronger environmental adaptability could be filtered out and combined intentionally. And when the technology is not associated with any mutagenesis technologies (theoretically the genetic essence is no different from the original parents), it essentially brings out new to speed up metabolism, and could consequently let the fusant show stronger activity and vitality, which may provide a solution to the aging or degradation of most commercial edible varieties.

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