Chapter 26
Biology and Molecular Approaches in Genetic Improvement of Cultivated Button Mushroom (*Agaricus Bisporus*)

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26.1 General Biology

26.1.1 Morphology and Life Cycle

Practically all the cultivated species of mushrooms (including *Agaricus bisporus*) belong to a subdivision of fungal kingdom designated as Basidiomycota. *A. bisporus* is placed in the order Agaricales of Basidiomycota, and are characterized by carrying sexual spores externally on a structure called basidium (pl. basidia).

The vegetative phase of *A. bisporus* is mainly underground and made up of filaments called hyphae, which branch in all directions and constitute the mycelium. The reproductive phase is above ground and constitutes the fruiting body. Fruiting bodies may attain varied shapes in mushroom. However, the most common type of fruiting body, met mainly in Agaricales and to a lesser extent in other groups, is an umbrella type, consisting of Pileus (pl. pilei) or cap, supported by a stipe (stalk). The fruiting body of *A. bisporus* (Fig. 26.1) also consists of a pileus (cap) supported by a central stipe (stalk). The basidia are borne in a palisade-like layer called the hymenium on the lower surface of pileus, known as hymenophore. The hymenophore appear in the forms of gills or lamellae, which are radiating plates with hymenium covering the two faces.

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Fig. 26.1 Mushroom fruiting body

The distinguishing features of *A. bisporus* fruiting body are: Chocolate-brown spores (as seen in spore print), gills not attached to the stipe (free) and a distinct ring on the stalk (stipe) termed as the 'annulus'.

The life cycle starts with the germination of spore discharged from basidium. *A. bisporus* is characterized by all basidia bearing only two basidiospores, as opposed to the four produced in other *Agaricus* species. On germination, spores of *A. bisporus* produce a mycelium, which is characteristically fertile. In the case of all the other four spore species of *Agaricus* mycelium arising from uninucleate spore, they are in the first instance sterile, and only on mating with a compatible homokaryotic mycelium produce a fertile diakaryotic mycelium. Mycelium in *A. bisporus* is septate, and each of the cells contains all necessary information for independent growth. If a mycelium is fragmented, each fragment can regenerate to form a new colony. All the cells are multinucleate, as opposed to most of the other basidiomycota, where the cells are uninucleate or binucleate. Mycelium in *A. bisporus* does not bear any clamp connection, which is characteristic of dikaryotic mycelia of the majority of Basidiomycota.

Mycelium developing from germinating spore continues to grow as long as the conditions are favorable. It ramifies, absorbing food from the substrate, until ready to fruit. Fruiting is initiated by a change in the environment. The fruiting body begins as a tiny knob of tissue arising from the underground intertwining mycelium, usually referred to as pinhead. These hyphal knots increase in size and form the button stage. As the development proceeds, the differentiation of pileus and stipe takes place. In further development, very thin radiating plate-like structures known as gills or lamellae are formed on the undersurface of the pileus. The gills bear

Fig. 26.2 Section of gills and hymenium showing the basidium and basidiospores of *Agaricus bisporus*

spores and are covered by a structure called veil. Veil extends from the margin of the cap to the stalk.

On further development, the veil breaks, exposing the gills and allowing the spores to deposit on the surface below. The broken portion of the veil forms a ring on the stalk which is called annulus. A section of the gill (Fig. 26.2) under the microscope shows that the center of the gills is made up of bundles or of mycelial threads known as trama. Towards the outside of the trama, the cells form a thin layer of short cells called the sub-hymenium. The sub-hymenium gives rise to long club-shaped cells, which are at right angles to the surface of gills and are known as basidia. Basidia in *A. bisporus* have two spine-like projections, the sterigmata, on which basidiospores are borne. Interpersed in the basidia are many sterile cells known as cystidia. The two together constitute the hymenium.

There is a constant and progressive reduction in the number of nuclei per cell from the base of the stipe upwards, ultimately reduced to two in the young basidia. These two nuclei later fuse in the basidium, and the diploid nucleus is formed. The diploid nucleus undergoes meiosis, giving rise to four haploid nuclei. In *A. bisporus* these migrate in pairs to the two basidiospores, in contrast to most other mushrooms, where a single nucleus passes on to each of the four basidiospores, and on discharge starts the cycle again.

26.1.2 Taxonomy and Nomenclature

The genus *Agaricus* belongs to family Agaricaceae in the order Agaricales of Basidiomycota. The family Agaricaceae is characterized by having a central stipe, pileus ordinarily readily separable from the stipe, lamellae free or nearly so and easily separable, spore deposits in some shades of chocolate color to blackish, and partial veil present, leaving more or less distinct annulus on the stipe. The genus *Agaricus* in addition, to the above family characteristics, possesses fibrillose cuticle on the pileus, with fibrils more or less radially arranged and at times broken into squamules (Smith 1978).

There is no taxonomic monograph on the genus, and no consensus on the number of species (Elliott 1985a). Singer (1975) recognized 35 species worldwide. However, other workers (Elliott 1985a) have put the number of species from 40 to 100. Three groups are generally recognized within the genus; two major groups, *Rubescentes* and *Flavescentes* have been differentiated on the basis of color reaction of the broken or cut flesh. In *Rubescentes,* the cut flesh turns more or less red, and in *Flavescentes* the cap turns more or less yellow when touched. There is a third minor group, also containing species with delicate fruiting bodies (Elliott 1985a).

Though there are a number of edible species in the genus, commercially cultivated species are only two — *A. bisporus* and *A. bitorquis*. Both of these belong to the *Rubescentes* group. As stated previously, of the two, the major volume of production is in *A. bisporus* only; *A. bitorquis* only came into commercial cultivation only during the 1970s and has only been cultivated to a minor extent. The production of *A. bisporus*, referred to earlier, constitutes about 37% of total world production of all cultivated species. It has been intensively investigated in all its aspects, cultivation having started in the West over three centuries back.

The nomenclature of *A. bisporus* has been changing. The cultivated white button mushroom was referred to as *Psalliota campestris* in earlier books. However, subsequently it was transferred to genus *Agaricus* for taxonomic reasons and appeared as *Agaricus campestris*, and was so till recent times. Subsequently, the mushroom in commercial cultivation was found distinct from *A. campestris* in a number of characteristics, especially two-spored basidia. Lange (Smith 1978; Hayes 1978) described the two-spored species as *Psalliota hortensis* Lange, but this was renamed *A. bisporus* (Lange) Singer by the International Botanical Congress at Paris in 1954. In some publications (Smith 1978) it has been referred to as *A. bisporus* (Lange) Imbach, based on Imbach's description, which is current in American literature. In Europe and most other countries the name *A. bisporus* (Lange) Singer based on Singer's description is adopted. *A. campestris*, the common field mushroom, is also very popular with mushroom-eaters.

Hayes (1978) has reproduced Singer's description. The salient points are given below:

A. bisporus (Lange) Sing. *Lilloa* 22: 431 (1951). *Pileus*: pure white to stramineous, or brown, almost naked to finely and almost to quite appressedly squamulose (finely scaly), dry, with thick projecting margins, the latter some times fibrillate-serrate or crenate, convex then convex with flattened center or becoming entirely flat in old specimens, mostly 35–100 mm wide. *Lamellae*: whitish, then a beautiful pink, finally concolorous with spore print, close to crowded, narrow or moderately broad, free, edge fimbriate.

Spore print: 'Sepia', close to 'mummy brown'.

Stipe: White or whitish, annulated, solid in age becoming hollow, equal or slightly tapering upwards or with a slight bulb at the base, 30–120 × 10–18 mm. Basal white rhizomorph often very distinct of variable diameter, veil superior.

Context (flesh): white when fresh, becoming red by auto oxidation, pinkish on exposure.

Odor: at first very pleasant and weak, eventually slightly acidulous or flesh.

Basidia: 20–28 × 6.7–7.7 μ, one-, two- or three-spored, almost all or the majority two-spored. Basidiospores 6.3–8.5 × 5.5–6.8 μ, short-ellipsoid, smooth, without a distinct germ-pore, brown.

Wild races are found on soil rich in nitrogen, spontaneously in hot beds, green houses, near manure or road sides, among scattered horse manure, in gardens and parks, etc. Fruiting from May to early November in northern temperate zone. Natural geographical area probably all over the northern hemisphere, outside the tropics and the Arctic.

Singer (1961) distinguishes three varieties of the species, namely: (1) var. *bisporus*, the type variety with a brown pileus but only rarely cultivated, (2) var. *avellaneus* (Lange) Sing. with a pale brown pileus that corresponds to the 'Brown' variety in cultivation, and (3) var. *albidus* (Lange) Sing., with a white pileus in youth which becomes cream as it matures and corresponds to the cream-colored varieties of cultivation.

It is commonly accepted, however, that all of the white strains now in cultivation were originally selected from natural color mutations and by a continuous process of selection. A wide range of strains are now available with different growth characteristics.

Some taxonomists are of the opinion that the correct name of the white button mush room is *Agaricus brunnescens* Peck on a priority basis. However, in view of the widespread usage of *A. bisporus*, the changeover has not been effected (Kapoor 1989).

In view of widespread commercial cultivation of the species, efforts have been made over the years to develop new strains with desirable characteristics. To augment these efforts, the *A. bisporus* recovery programme (ARP) (now named as *Agaricus* Resource Programme) was founded by Dr. Rick Kerrigan of Sylvan Research, USA 1988 (Rai and Ahlawat 2002). More than 206 *A. bisporus* strains, including the wild ones, are available with ARP. These were collected from spawn companies and research institutes all over the world, and have been identified using isozyme marker techniques. They are an excellent source of genetic variability. As a part of this effort, Callac et al. (1993) reported a tetrasporic variety from the Sonoran desert of California. These authors described a new variety of *A. bisporus* named as *A. bisporus* var. *burnettii*. This differs from the earlier variety designated as var. *bisporus*, in the sense that most of the basidia of this variety are tetrasporic, while the var. *bisporus* had 81% bisporic, 18% trisporic and 1% tetrasporic basidia; var. *burnettii* had 85% tetrasporic, 14% trisporic and 1% bisporic basidia. The tetrasporic variety holds promise for simplifying the process of homokaryon isolation, particularly for application in a hybrid breeding programme.

26.1.3 Nutritional Requirements

A. bisporus being a heterotrophic organism, like all other saprophytic fungi, depends for its nutrition and subsequent metabolism on carbon compounds that have already been produced by green plants. In addition to the carbon compounds (often sugars), nitrogen compounds (either in organic or inorganic forms), essential mineral nutrients (calcium, magnesium, potassium, phosphorus, sulfur, copper, iron, etc.), vitamins (thiamine and biotin), and growth factors are essential for successful growth of the mushroom. Hence, a thorough knowledge of nutrition is essential for successful commercial cultivation procedures. Wood and Fermor (1985) have provided an exhaustive review of the nutritional requirements of *A. bisporus* both in pure culture and in commercial growth substrates. The salient points are presented below.

26.1.3.1 Carbon

Investigations on carbon nutrition in pure culture for both mycelial growth and fruiting have been carried out and reviewed by a number of workers (Wood and Fermor 1985). The results obtained have led to the conclusion that in general, glucose, fructose and xylose are good carbon sources, and other sugars and organic acids less good. The ability to degrade and utilize various higher molecular weight carbohydrate polymers, particularly those found in green plants, has also been investigated. Starch, glycogen, mulin, xylan, cellulose (filter paper) and pectin are utilized to varying degrees. Utilization and degradation of cellulose and hemicellulose have also been investigated, and found to provide good growth (Hayes 1978; Wood and Fermor 1985; Mohammadi Goltapeh and Pourjam 2005).

Studies concerning the nutrition of *A. bisporus* in composted wheat straw have demonstrated a preference for utilizing the lignin and protein fractions of compost, but cellulose and hemicellulose fractions could also be utilized (Wood and Fermor 1985). It has also been shown that supplementation of compost with lipid- and protein-rich materials such as various seed meals, oil or defatted seeds leads to increased crop production (Hayes 1978; Wood and Fermor 1985; Mohammadi Goltapeh and Pourjam 2005).

26.1.3.2 Nitrogen

A. bisporus, like all other fungi, requires a nitrogen source. Suitable nitrogen sources include urea, a range of ammonium salts and many amino acids — asparagine, alanine and glycine are especially good sources (Hayes 1978). Various types of protein are efficiently utilized by *A. bisporus* (Wood and Fermor 1985). The nitrogen sources available to *A. bisporus* in the compost are lignin–nitrogen fraction

of compost and microbial protein synthesized during the composting process (Wood and Fermor 1985).

26.1.3.3 Essential Elements

Four macronutrients — magnesium, phosphorus, potassium and sulfur — and five micronutrients — boron, iron, manganese, molybdenum and zinc — are required for optimum mycelial growth of *A. bisporus*. However, in *A. bisporus*, in addition to the above, calcium is also required and has been shown to fulfill an important physiological role in the medium (Hayes 1978; Wood and Fermor 1985). Traditional horse manure compost at spawning and wheat straw can provide for all mineral nutrients, and in a properly formulated compost, none is likely to act as a limiting factor.

26.1.3.4 Vitamins and Growth Factors

Biotin and thiamine have been shown to be required for mycelial growth (Hayes 1978). However, microbial flora in the compost produce B-complex vitamins, and composts (both horse manure and synthetic) would not be vitamin-deficient (Wood and Fermor 1985). Growth-promoting activity has been shown with the addition of indole acetic acid, esters of oleic and linoleic acid and amino acids — phenylalanine, methionine and proline (Hayes 1978).

26.1.3.5 Role of Microbial Biomass

Microbial activity converts the components of straw and manure into mushroom compost. The total count of these micro-organisms (both living and dead) in composts has been termed microbial biomass. Various workers have suggested a role for this biomass in the nutrition of *A. bisporus*. Wood and Fermor (1985) have reviewed these studies. Biomass could also act as a concentrated source of nitrogen and minerals.

26.1.4 Environmental Requirements

Environmental parameters have a definite role to play in commercial production of *A. bisporus*. At every stage in the production process, starting with composting, the environment has to be carefully manipulated and controlled. In seasonal or cottage-scale cultivation, a favorable environment prevailing during the season is exploited and obtained by manipulation of growing conditions. However, in highly controlled conditions, a precise environment is simulated for crop-raising, and in some farms

computerization has been introduced to automatically maintain and control the environmental parameters in the cropping room and in bulk chambers.

26.1.4.1 Temperature

An optimum temperature is essential for growth and reproduction of any living organism, including the mushroom. In most of the cultivated types of mushrooms (including *A. bisporus*), the optimum temperature for vegetative growth is different from that required for fruiting (sexual phase). The optimum temperature for mycelial growth in *A. bisporus* is in the range of 23–25°C (air temperature). Limited mycelial growth takes place at temperatures $\leq$3 and $\geq$30°C. However, for fruiting, temperatures are in the range of 15–17°C. At temperatures $\geq$20° and $\leq$14°C, growth in the developing buttons is slowed.

26.1.4.2 pH

Though vegetative growth is possible over an extended range of 3.5–9.0, optimum pH is around 6.0. A pH of 6.8–7.2 is considered ideal for fruiting, which is maintained in compost and casing for commercial cultivation.

26.1.4.3 Light

Light does not play any role in vegetative growth or fruiting of *A. bisporus*. However, exposure of even smaller periods of light in off-white strains results in browning of the mushroom (Kapoor 1989).

26.1.4.4 Moisture

Moisture content of the substrate and relative humidity of atmosphere in the crop room are of prime importance in growth and reproduction of *A. bisporus* as in other fungi. Fifty-five to 65 percent of water in the substrate has been found to be appropriate for initiating fruiting in *A. bisporus* (Manchere 1980). Flegg found that the best growth occurred when the water content of the compost ranged between 55% and 70% (Flegg and Wood 1985).

26.1.4.5 Relative Humidity

Relative humidity of the atmosphere in the crop room plays a vital role during growth and reproduction of *A. bisporus*, especially during pinhead formation.

RH levels of 85% and 95% have been found to be optimum during initiation and development of fruiting bodies and cropping, respectively.

26.1.4.6 Aeration

Relative concentration of oxygen and carbon dioxide, and presence or absence of toxic gases in the atmosphere, affects the growth and fruiting in *A. bisporus*. In consequence, the role of aeration assumes great importance, and is to be carefully controlled at various stages. Growth in pure culture is not affected at oxygen concentrations ranging from 21% to −0.6% (Hayes 1978).

The influence of carbon dioxide has been investigated, and mycelial growth is reduced at about 2% and is totally inhibited at 32%. Initiation of fruiting occurs, preferentially at a concentration ranging from 0.03% to 0.1%, and 0.5% to 1.0% is high enough to inhibit the premordia formation (Kaul 1978; Flegg and Wood 1985).

26.1.5 Sexuality and Breeding

The nature of sexuality and breeding strategies adopted in edible mushrooms has been comprehensively dealt with in an earlier publication (Kaul 2002). An attempt will be made here to present in brief the situation in *A. bisporus*, with special focus on the molecular techniques used in genetic improvement of the crop.

The yield and quality of mushrooms produced by commercial growers is mainly determined by two factors: (1) the genetic make-up of the strain, and (2) the environmental condition in which the strain is grown. The genotype of the strain has a big role to play in the quality and quantity of mushrooms obtained, and any improvement is welcome. The goal of the mushroom breeder is the assembling of the best combination of genes into one individual stock for production of mushrooms of high quality. To achieve this, it is necessary to have a basic understanding of the sexuality of individual species and possible breeding strategies.

The life cycle, pattern of sexuality and sexual mechanism are three parameters of sexual behavior in *A. bisporus*. The life cycle in *A. bisporus* differs from nuclear phases of a typical basidiomycota in having a heterokaryotic (dikaryotic phase) originating directly from germinating spore. The majority of the basidia are bispored, having two compatible nuclei each, which on germination give rise to heterokaryotic dikaryon directly without plasmogamy (union of homokaryotic mycelia). In rare cases the basidia are four-spored, where spore gives rise to homokaryotic mycelia. Dikaryon in *A. bisporus* is multinucleic, unlike most other edible mushrooms. As in other basidiomycota, dikaryon is the predominant phase, leading to the production of fruiting bodies. Subsequently, the transient diploid phase is initiated by karyogamy (union of nuclei) in basidia. This is immediately followed by meiosis, resulting in four nuclei in the basidium. These, unlike those in other edible mushrooms, are transferred to two spores in pairs in *A. bisporus*.

Two basic patterns of sexuality recognized in Basidiomycota are: (1) homothallism, where mycelium from a single germinated spore is self-fertile, and (2) heterothallism, where a single basidiospore germinates to give rise to monokaryon, which must fuse to produce a fertile mycelium. However, the pattern in *A. bisporus* has been designated as secondary homothallic. In this pattern, though single spores are fertile, each spore has two compatible nuclei. Heterothallism here is concealed by the two-spored nature of basidia.

Patterns of sexuality are in general controlled by two mechanisms: (1) the distribution of four post-meiotic nuclei to the basidiospores, and (2) genetic factors of a mating system known as the incompatibility system, which may be either unifactorial or bifactorial. In *A. bisporus* in most cases two spores receive two nuclei: each of these forms a basidium which is of a different mating type and which has unifactorial control.

26.1.5.1 Genetic Improvement

The main hurdle in serious scientific work on the cultivated mushroom has been the mystery about the life-cycle of fungi. Discovery of the aseptic technique and subsequent understanding of the life cycle opened up the possibilities of improvement in mushroom crops. The first germ-free spawn was made in 1894 at Pasteur Institute, Paris, and Duggar perfected the method in USA in 1905 (Singer 1961).

Though cultivation of *A. bisporus* is over three centuries old, still very little has been achieved in respect of improved strains. Even the appearance of the white strain in *A. bisporus* was a result of spontaneous mutation in a population of cream fruiting bodies. The cultivation technology has been thoroughly investigated, and taken to the level of computerization on large-scale commercial farms. However, the breeding programme has not advanced much. This has been mainly because of incomplete knowledge of the sexuality of this mushroom. This was elucidated only about two decades back, and Elliott (1985b) has traced the historical development in the understanding of sexuality in *A. bisporus*. *A. bisporus* was classified in the secondary homothallic group, with an unusually complex breeding system.

A. bisporus lacks two of the diagnostic features of Basidiomycota. Firstly, it has two-spored basidia rather than four-spored ones on its gills, and secondly its vegetative mycelium lacks clamp connection. The fertility of single spore (binucleate) isolate is because many spores receive nuclei from basidium which are of different mating types. Horgen (1992) is of the opinion that there appears to be a controlled biological mechanism which ensures that the basidiospores contain nuclei of a compatible mating type. The formation of two binucleate spores on each basidium is the normal pattern of development in this species, but occasionally (about 20%) a basidium may bear three or four spores. The latter receive only a single nucleus and are infertile. Secondary homothallism of *A. bisporus* where a single spore is fertile due to having two compatible nuclei has also been substantiated by genetic analysis conducted by Raper et al. (1972).

The fertile heterokaryotic mycelium that normally develops from the germinated basidiospore in *A. bisporus* is morphologically indistinguishable from the exceptionally occurring self-sterile homokaryotic mycelium. The cells of both mycelia are multikaryotic. The cells also lack clamp connections, which are a feature of fertile heterokaryotic mycelium in most other species. The creation of a fertile heterokaryon by crossing two compatible self-sterile homokaryons can therefore be detected only through the tedious process of a test for fruiting. This fact, together with the extreme mechanical difficulty of isolating the rare self sterile, cross fertile homokaryons, has presented serious problems in breeding in *A. bisporus*. The available systems for strain improvement in *A. bisporus* are: selection, breeding and gene transformation (Sodhi et al. 1997). Selection was the only course followed for obtaining improved strains prior to 1970.

26.1.5.2 Selection

The selection procedure in *A. bisporus* (as in other edible mushrooms) has been utilized either in introduction and screening of new cultures from foreign countries, or in screening the existing commercial strains. The three procedures usually followed are tissue culture, multispore, or single spore.

Tissue culture taken from the stem or cap of a mushroom has been the oldest method of raising cultures from the wild type. Tissue culture raised from phenotypically healthy looking fruiting bodies for strain improvement has been suggested by some authors for improvement. However, the method has not been found to be of much promise in *A. bisporus* (Yadav et al. 2002).

In the multispore method, a spore print is obtained from a suitable fruiting body and mass of spores germinated together. Elliott (1985b) states that most commercial strains in *A. bisporus* prior to the release of hybrids were obtained by this method. However, other authors are of the opinion that multispore culturing does not generate significant variability in terms of genetic improvement (Mehta and Bhandal 1994).

The single-spore selection method is based on the premise that about 70% of spores produced in *A. bisporus* are fertile, and that selection can be practiced among these fertile spores. It has been widely followed for developing superior cultivars in *A. bisporus*. Isolation is a time-consuming process, but the selection of superior strains out of single-spore isolates is the most acceptable method in *A. bisporus* (Sodhi et al. 1997). However, other authors maintain that the method has perhaps a short-term role in strain improvement in *A. bisporus*, and only limited gains for genetic improvement are expected (Elliott 1985b; Mehta and Bhandal 1994).

26.1.5.3 Hybridization

Subsequent to elucidation of the sexual cycle by Miller (1971) and Elliott (1972), efforts have been made to obtain improved stocks of *A. bisporus* by hybridization. It involves mating (anastomosis) of self-sterile and compatible homokaryotic lines.

Commercial successful hybrid spawn was achieved first in the Netherlands when Fritsche (Elliott 1985b) released such spawn under codes U_1 and U_3 in 1981. These hybrids were the result of a breeding programme to combine the desirable qualities of the off-white and pure white spawn types. The strains are highly productive, with better size, and have dominated commercial markets ever since. Another hybrid obtained with this procedure has been from Taiwan (Elliott 1985b). Other hybrids available are Amycel 208 and NCH-102, from USA and India respectively (Yadav et al. 2002).

The steps involved in the breeding programme of *A. bisporus* are as follows:

1. *Selection of parent fruit bodies* with desired characteristics and collection of spore print (parents with different characteristics are selected).
2. *Single spore isolation*. Spore prints obtained are serially diluted and finally plated. The single spores are located under microscope and transferred to plates with a suitable medium.
3. *Testing of single spore isolates for homokaryotic nature*. The majority of single spores are heterokaryotic, with a small minority being homokaryotic. Traditionally, the criteria of colony morphology (appressed type), slow mycelial growth and non-fruiting were used for identification of homokaryons. The procedure is quite cumbersome. However, modern tools of molecular biology — isozyme (allozyme) analysis, electrophoresis, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) — are now available for homokaryon isolation in *A. bisporus*. With a combination of isozyme analysis, RFLPs and RAPDs, the identification of homokaryons is now a routine process in the *A. bisporus* breeding programme (Loftus et al. 1995).
4. *Selection for homokaryons* has also been made easy with the discovery of tetrasporic variety var. *burnettii* (mentioned earlier). The majority of basidia (85%) in the variety having four spores, isolation of homokaryons is easy.
5. *Homokaryons thus selected* are crossed in various combinations to locate compatible single-spore isolation, and in the success of such a cross-hybrid follows. . .
6. *Identification of hybrid*. The recognition of hybrids again poses a major problem in *A. bisporus*, unlike many in other edible mushrooms, as no clamp connections are formed. Identification was traditionally made by the microscopic observation that compatible homokaryons show heavier growth in the zone of confrontation. A fruiting test was necessary for confirmation. However, molecular markers are now mainly utilized for identification of hybrids.
7. *Grow out tests* of the hybrids developed in this way, for expression of the desired characteristics of the parents.

26.1.5.4 Molecular Markers

Alozyme (isozyme) markers. The first genetic test for homokaryons and hybrids which became available to the breeder was isozyme analysis. Isozymes are different

forms of the same enzyme, differing in electrophoretic mobility as a result of allelic differences in a single gene. In isozyme analysis, mycelium is ground up and the cellular extract is run on electrophoretic gel. With the use of an enzyme-specific strain, homokaryons can be distinguished from heterokaryons. Staining gives characteristic banding patterns, and strains with different banding patterns can be crossed: hybrids are recognized by novel banding patterns (Elliott 1985b). More than a dozen isozyme markers have so far been identified in *A. bisporus* (Rai and Ahalawat 2002).

Auxotrophic markers. Auxotrophs are strains which have additional nutritional requirements for growth, and are the result of nutritional changes in individual genes (Elliott 1985b). These can be used to make hybrids. If a cross is made which is deficient for amino acid praline and capable of making methionine is mated with a strain deficient for methionine but capable of making praline, a hybrid will be produced which is capable of growth on a medium lacking both these amino acids, and can be easily recognized. Unfortunately, auxotrophs are not easily available due to the masking effect of multikaryotic nature of *A. bisporus* cells. Elliott (1985b) states that this procedure will not have an important role in any hybridization programme.

Resistance markers. In this, a strain resistant to a fungicide can be intermated with another strain resistant to another fungicide, and their hybrid can be identified by growing the intermated culture on a medium containing both fungicides. Several fungicide-resistant mutants of *A. bisporus* have been produced using UV mutagenesis, and are being evaluated for their insensitivity to fungicides during cropping (Elliott 1985b).

RFLP markers (restriction fragment length polymorphism). In the mid 1980s, more powerful DNA-based techniques became available, and RFLP is one of them. RFLP markers show heritable differences in the length of DNA fragments which are generated when DNA is digested by a restriction endonuclease. If the DNAs of different strains are cut with the same restriction enzyme, any natural variation or polymorphism in the position of the site at which the enzyme cuts will result in DNA fragments which differ in length. DNA fragments generated by restriction endonucleases are separated by gel electrophoresis, and made single-stranded by their transfer onto a nitrocellulose-based membrane for hybridization (Rai and Ahlawat 2002). These can be visualized using labeled DNA probes (Challen et al. 1991; Clutterbuck 1994). RFLP markers have been used both for identification of homokaryons and confirmation of their hybrids in *A. bisporus* (Yadav et al. 2002). RFLP analysis measures genetic differences at the most fundamental level in an organism — the DNA. Some of the limitations in the use of these markers are the high recurring cost and the requirement for a large quantity of pure DNA (5–10 μg).

RAPD markers (randomly amplified polymorphic DNA). Another method in widespread use to-day is RAPD analysis. RAPD is basically based on the amplification of random DNA segments by polymerase chain reaction. The polymerase chain reaction (PCR) is the pioneering technique for *in vitro* amplification of nucleic acids. The PCR produces greater than 10^6-fold amplification of a target DNA sequence in 1–2 h (Keller and Manak 1993). The requirement for a small

quantity of DNA, fast speed and ability to handle multiple numbers of samples at a time, makes this technique more user-friendly than RFLP. RAPD markers have been utilized for homokaryon identification and confirmation of hybrids generated by crossing two compatible homokaryons. However, RAPD analysis is considered less robust than RFLP analysis — RFLP results being more consistent and reproducible from laboratory to laboratory (Loftus 1995).

RAPD and RFLD DNA marker systems are the means by which DNA fingerprints are made. DNA fingerprints are also made for patent applications, and being unique to an individual mushroom strain, they protect the strains from theft by competing spawn companies.

26.1.5.5 Genetic Engineering

Genetic engineering, also referred to as transformation, is the technology that permits the transfer of specific genes within and between species in a way that is both precise and simple. It involves the isolation, cloning, sequencing and insertion of desirable gene(s) into the genome of a target organism to obtain expression of the desirable trait. The birth of recombinant DNA technology or genetic engineering occurred in the 1970s when a group of scientists succeeded in splicing viral and bacterial DNAs in test tube (Romaine 2002).

The method is based on the ability to cut DNA molecules precisely into specific pieces, and to recombine those pieces to produce new combinations. The procedure depends on the existence of restriction enzymes that break DNA at sites where specific nucleotide sequences occur. Any two fragments produced by the same restriction enzymes can be joined with one another in this way, using a sealing enzyme called ligase (Raven et al. 1986). Transfer of genes is affected through vector, electroporation or ballistic gun system.

Vector transformation is the preferred route, and if it fails then other methods are used. In the ballistic gun method, small fragments of DNA with desired genes are coated on a tungsten chip and bombarded onto any tissue of mushroom with very high speed.

In vector-mediated transformation, plasmids are used. Plasmid is a small fragment of DNA that exists free in the cytoplasm of a bacterium and can be integrated into and then replicated with a chromosome. These are common in bacteria but rare in Eukaryotes. Bacterial plasmid (T_i) associated with the crown gall bacterium *Agrobacterium tumefaciens* has been found useful in inserting foreign gene into plants and has subsequently been used in fungi too. Significant advances in the development of transgenic crop plants have been made. We have ‘golden rice’ where a gene from daffodil has been introduced to make the rice rich in beta carotene, and Bt cotton where insect-resistant genes have been imported from *Bacillus thuringensis* bacterium.

With the discovery in 1995 that *A. tumefaciens* can also operate in yeast fungi, ways were opened for its use as a vector in filamentons fungi. Since then, a lot of

work on genetic manipulation of *A. bisporus* and other mushroom species has been done through this vector. Romaine (2002) has provided a review of these efforts. He also outlined the steps in the *Agrobacterium*-mediated gene transfer method. These are:

1. Gill tissue is taken from mushroom with intact veils.
2. This is cut into 2–5 mm pieces.
3. The tissue pieces are vacuum infiltrated with a suspension of *Agrobacterium* carrying an antibiotic (hygromycin)-resistant gene.
   (a) The tissue pieces are placed on a medium amended with hygromycin.
   (b) Within several weeks the mushroom cells which received the resistance gene grow into visible culture.

Though transformation attempts have been successful in many species, e.g., *Coprinus* sp. and *Schizophyllum commune*, no significant success has been achieved in *A. bisporus* so far (Sodhi et al. 1997; Rai and Ahlawat 2002 and Mehta and Bhandal 1994). Efforts are, however, ongoing, and a long list of desirable clonable genes from *A. bisporus* and possible useful genes from other sources for incorporation in *A. bisporus* have been identified (Sodhi et al. 1997). Challen et al. (2000) have reported preliminary success in transformation of a number of edible mushrooms — *Agrocybe aegerita*, *A. bisporus*, *Lentinus edodes*, *Pleurotus ostreatus* and *Volvariella volvacea*. Though *Agrobacterium tumefaciens*-mediated transformation was successful in *A. bisporus*, yet further refinement is needed before transgenic strains can be developed.

Sodhi et al. (1999) have also constructed genomic libraries for *A. bisporus*. A genomic library is a set of recombinant clones which represent the complete DNA present in an individual organism, and its construction is a step toward mushroom strain improvement.

Loftus et al. (1995) maintained that the potential of genetically engineered mushroom is immense, and is readily apparent in three main areas: virus resistance, improved shelf life, and improved compost utilization.

26.1.5.6 Mutation Breeding

Mutagenesis is attempted to create new variability for the selection and hybridization programme. A natural mutant was responsible for isolation of the white strain of *A. bisporus* from the cream strain in 1927, which is in widespread commercial culture (Kumar 1997). Though success in obtaining higher penicillin yielding strains of *Penicillium* has been achieved by cycles of mutation and selection, no such success has been achieved in *A. bisporus*. Mutagenesis may have value in production of markers for identification of hybrids (Elliott 1985b).

26.1.5.7 Protoplasting

Protoplasts are created when the cell wall is digested away from mushroom mycelium, leaving 'naked cells'. Protoplasts can be plated out and cell walls regenerate. If a heterokaryon is protoplasted, a proportion of the mycelial colonies from the regenerated protoplasts will possess one nucleus, giving rise to homokaryons. Protoplasts are thus useful as a tool for isolating homokaryons in *A. bisporus*.

Protoplasts are also useful in genetic engineering experiments, whereby DNA can be introduced into the cell without the constraints provided by the cell barrier. Protoplast technology involves the following sequential steps (Yadav et al. 2002):

1. Isolation of protoplasts using cell wall digestive enzyme.
2. Fusion of protoplasts with polyethylene glycol and $CaCl_2$.
3. Regeneration and evaluation of somatic hybrids.

The fusion of protoplasts can also be achieved using the electrofusion technique. There has been no significant achievement in the improvement of *A. bisporus* strains by this method either.

Amycel Strains

A number of laboratories involved in the production of improved strains are making use of new technology. Amycel/Spawnmate biotechnological group research laboratories in Watsonville, CA, USA, has a major programme in strain improvement, and Loftus et al. (1995) have described in detail the technologies in use, achievements made and emerging technologies of the future. Systematic breeding of new mushroom cultivars through a combination of molecular breeding techniques, specific traits identified in wild *A. bisporus* and backcrossing strategy (similar to that used in plant breeding) is being attempted by Amycel.

Loftus (1995) concludes that future commercial mushroom spawns will be created through a range of new technologies (which include genetic engineering); novel strains protected through patents will be available to growers, and the days of dominance of the off-white hybrid strains are numbered.

26.2 Trouble Shooting

- Morphologically, *A. bisporus* resembles many poisonous mushrooms such as Amanita. Hence the identification of wild collections must be done by an expert, or otherwise the strains should be obtained from an authentic source.

- The cultivation of the mushroom is generally been done on composted wheat straw, so the formulation must be done in such a way that the carbon:nitrogen ratio is set at 35:1 at the start of the composting process, and the final C:N ratio must reach 18:1 after the microbial succession. Poor production or disease and pest infestations are generally the results of an improper C:N ratio at the end of the composting. Hence if the C:N ratio is higher than 18:1 at the end of the composting, the substrate should be composted for a few more days until it reaches 18:1.
- The fungal mycelium is very sensitive to ammonia, which is a byproduct of the composting process. Presence of even small amounts of ammonia may result in poor mycelial growth and reduced yield of the mushroom. To avoid such conditions, the presence of ammonia must be checked at the end of the composting process, either by smelling or by ammonia checking strips, and if even small traces of ammonia are detected, the compost must be reconditioned to release the traces of ammonia.
- Environmental requirements also affect the production of the mushrooms; hence, the pH of compost must be adjusted near to 7.0 with the use of gypsum. The temperature is another environmental consideration for the good yield of the mushroom; hence, the optimum temperatures for mycelial growth and fruiting must be maintained properly. The requirements of oxygen of the fungus during mycelial run and fruit body formation are different. The mushrooms require a high concentration of CO_2 during the mycelial run phase, while during fruiting a high concentration of oxygen is required to switch over from the vegetative to the reproductive phase of the fungus. As the mushroom contains 90% water, and this water is taken from compost/casing at the time of fruiting, hence the moisture content of the compost beds must be maintained close to 65% by regular water spray and maintaining relative humidity in the cropping rooms.
- Breeding new varieties in *A. bisporus* is a difficult task, because it is secondarily homothallic in nature, producing homokaryons as well as heterokaryons. Hence, before selection of the single-spore isolate for hybrid breeding, it is a must to ascertain the homokaryotic nature using molecular markers, and also the probable hybrids must be confirmed by DNA markers as well as fruiting trials.
- The hybrids produced in this way are always prone to segregate for traits; hence, the hybrid strains must be maintained vegetatively to maintain their hybrid nature, and also the culture medium must be alternated between poor and rich nutrient levels to maintain the vigor of the strain.

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