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Mutation Breeding of African Nightshade (*Solanum* section *Solanum*)

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ABSTRACT

African nightshades (*Solanum nigrum*-related species) are some of the most widely consumed traditional leafy herbs and vegetables, particularly in Africa and South-East Asia. The leaves contain high levels of vitamins (especially A, B and C), mineral fibres (such as iron, calcium and phosphorus), carbohydrates and proteins. They also contain phenolics and alkaloids, such as nicotine, quinine, cocaine, and morphine, which are known for their medicinal attributes. With the realization of their high nutritional, medicinal and health benefits, the demand for these vegetables has been on a rapid and steady rise in the recent years. However, due to very low leaf yields that are considered uneconomical compared to other high-yielding and high-value horticultural crops, production of these vegetables remains on a small scale. Prolific early flowering and excessive fruit- and seed-set, which compete with leaf production, are the main limiting factors on leaf yields. To eliminate or reduce fruit-set, hence competition with leaves, induction of male-sterility is probably one of the most immediate options. The main challenge that faces this strategy is propagation and maintenance of male-sterile lines. This review focuses on the mutation breeding for improved leaf yields of African nightshades with special reference to male-sterility. Aspects of propagation and maintenance of male-sterile lines are discussed.

Keywords: fertility restoration, leaf yields, male-sterility, mutagenesis, *Solanum nigrum*, vegetative-reproductive balance

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INTRODUCTION

A mutation is a sudden heritable change in the DNA of a living cell, not caused by the common phenomena of genetic segregation or genetic recombination. Mutations may occur in nature without intentional human intervention, and are said to be spontaneous. Spontaneous mutations may result from the activity of mobile genetic elements (transposons) that can move around to different positions within the genome of a single cell and affect the activity of the gene in which they are inserted (reviewed by Wessler 2006). Mobile genetic elements affect the gene function through various mechanisms. Retrotransposons, for example, move in the genome by being transcribed to RNA and then back to DNA by reverse transcriptase, while DNA transponsons move directly from one position to another within the genome using a transposase enzyme to "cut and paste" them within the genome, causing spontaneous mutations (Kidwell 2005). Most spontaneous mutations occur in very low frequencies e.g. 10^{-6} of an individual gene. Moreover, not all phenotypically observed variation refers to genetic changes. At the same time, not all the spontaneous changes in the DNA ultimately result in permanent changes of the DNA. Even if such changes would be permanent, they may not always result in visible or detectable effects (Ranel 1989). For example, there may be latent adaptive mutations in African nightshade that help the plants to survive in the wild, but these are not known so far. Besides, spontaneous mutations depend on chance and make breeding programmes considerably slow.

Although selection for economically useful spontaneous mutants still takes place with some level of success (See review by Ahloowalia *et al*. 2004), the purposeful induction of a specifically desired mutation at a specific time and place, and in a selected genotype for a selected purpose is a much more attractive option. The discovery that X-ray-in-

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duced mutations in *Drosophila melanogaster* (Müller 1927) and in *Hordeum vulgare* (Stadler 1928) led to the use of radiation-induced mutations for changing plant traits by plant breeders and geneticists. Mutation induction raises the natural mutation rates 10-100 fold, expanding the opportunity to isolate a higher number of mutants in a limited space. Today, induced mutations are ideal for augmenting natural variation in germplasm and as an alternative to hybridization and recombination in plant breeding. Mutations provide new starting material for the production of new cultivars and on the other hand they offer excellent tools for identifying new genes, for studying the nature of genes and their way of controlling biochemical pathways (Micke *et al*. 1990). The genetic variation from mutagenesis is different from that existing in germplasm collections or obtainable from crossing as it is not yet selected by nature or man and thus contains traits which were not favoured during evolution or previous plant breeding activities. Besides, genes for a desired trait may not be fit or may be tightly linked with undesirable genes so that recombination through hybridisation is rare or impossible. For example, genes causing pollen abortion cannot be transmitted gametophytically to future generations; consequently homozygous plants with all aborted pollen are lost in the cause of evolution. Although various types of male-sterility occur in flowering plants, there have been no reports of spontaneous male-sterile African nightshade plants.

Mutation breeding is the purposeful application of mutations in plant breeding. It offers good prospects for the domestication of promising underutilized wild species such as African nightshade, for agricultural or horticultural uses as well as for improving adaptation of recently introduced crops to unsuitable environments (Anon 1986). Mutagenesis has remained popular for close to a century because of its simplicity, technical and economic viability, applicability to all plant species and usability at small or large scales (Siddiqui and Khan 1999). More than 2000 plant varieties that contain induced mutations have been officially released for cultivation either directly as new varieties or used as parents to derive new varieties without the regulatory restrictions faced by genetically modified material (Maluszynski *et al*. 2000; Waugh *et al*. 2006). The main strategy in mutation-based breeding has been to upgrade the well-adapted plant varieties by improving a few desirable major yield and quality traits (Ahloowalia *et al*. 2004). Besides, the increased yield and enhanced quality of the novel varieties included several other components such as subsequent use for breeding, improved harvest index from heterosis in hybrid cultivars, response to increased agronomic inputs, and consumer preference.

INDUCTION OF MUTAGENESIS IN AFRICAN NIGHTSHADE

A physical or chemical agent that changes the genetic information (usually DNA) of an organism and thus increasing the number of mutations above the natural background level is called a mutagen. De Vries (1905) suggested the use of radiation to induce mutations. Müller (1927) working with *Drosophila* and Stadler (1928) working with cereals demonstrated that mutation frequency could be enhanced with the help of mutagenic agents like X-rays. Auerbach and Robson (1946) reported the use of chemicals such as mustard gas to be highly mutagenic. Since then a number of agents have been discovered that can increase the frequency of artificially induced mutations. The main mutagens available for induction of mutations include UV radiation, electro-magnetic waves such as X-rays, γ-rays and cosmic rays; fast moving particles such as α particles, β particles and neutrons; and chemical agents such as, alkylating agents, acridines, azides, hydroxylamides, etc. In general, ionising radiations such as X-rays and γ-rays are preferred over chemical mutagens because of their ease of application, good penetration and reproducibility, high mutation frequency and less disposal problems. The X-rays are obtained from X-ray machines by bombarding tungsten or molybdenum with electrons in a vacuum, whereas γ-rays are obtained from radioisotopes like Cobalt-60 and Caesium-137 in the γ chamber. The UV- radiations possess limited tissue penetrating ability {low linear energy transfer (LET)} and cause relatively little damage except after prolonged exposure as a result of which their use is restricted to pollen grains (Kovács and Keresztes 2002).

Mutagenic agents vary in their mode of action. UV light is absorbed by pyrimidines in DNA, causing adjacent bases on the same DNA strand to bond covalently to form pyrimidine dimers that subsequently cause errors during DNA replication. X-rays, γ-rays and cosmic rays may act directly on the critical targets in the cell (particularly, the DNA), or interact with other atoms or molecules in the cell, particularly water, forming radicals that break DNA strands and alter purine and pyrimidine bases (Keresztes and Kovács 1991). This radiolysis of water may affect temperature, pH and dilution of solutions by the presence or absence of oxygen (Diehl 1990). Changes in the DNA may include disruption of continuity of one or both strands, removal of a base and chemical alteration of a base, which changes its pairing properties causing gene mutations. Several mechanisms may be responsible for the occurrence of mutations after DNA damage. One mechanism may involve an alteration in the specificity of base pairing (e.g. de-amination of cytosine to uracil). The resulting mispairing leads to errors during replication and a mutation may be induced opposite to such lesions. Another mechanism may involve the loss of base pairing potential, leading to mutations (Kovács and Keresztes 2002). Fast moving particles such as fast neutrons have much higher LET and can physically 'punch holes' in DNA (Waugh *et al*. 2006).

Chemical substances have three general modes of action. One, base analogues such as 2-amino purine (resembling adenine) and 5-bromouracil (resembling thymine) with different hydrogen bonding properties get incorporated into DNA during replication. For example, 5-bromouracil if incorporated instead of thymine pairs with guanine (instead of adenine) causing the incorporation of cytosine into the daughter DNA strands during subsequent rounds of replication (resulting in A/T to G/C transitions). Two, intercalating agents such as ethidium bromide, proflavin and acridine orange slip between adjacent base pairs in DNA, reducing the fidelity of DNA replication and causing insertions, deletions or additions that induce frameshifts. Three, alkylating agents such as ethyl methane sulfonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU), deaminating agents such as nitrous acid and nitrosoguanidine and hydroxylating agents such as hydroxylamine are base modifying agents. EMS adds alkyl groups to the hydrogen-bonding oxygen of guanine to produce *O*-6 alkylguanine, which pairs with T (instead of C) and causes G/C to A/T transitions. Nitrous acid converts cytosine to uracil (by oxidative deamination), which forms hydrogen bonds with adenine rather than with guanine and causes A/T to G/C transitions. Hydroxylamine reacts with cytosine, converting it to a modified base that pairs only with adenine and results in C/G to T/A transitions (Waugh *et al*. 2006). Of the chemical mutagens, EMS has been quite useful in inducing point mutations in the genomes of a diverse range of plants largely because of its well established mode of action, which generates G to A and C to T transitions (Koornneef *et al*. 1982), and its effectiveness in inducing a high frequency of point mutations in a wide range of organisms without causing gross chromosomal abnormalities (Ahloowalia *et al*. 2004).

MUTAGEN DOSAGE AND MUTATION FREQUENCY IN AFRICAN NIGHTSHADE

Mutagen treatment causes complex genetic and physiological damages. The first generation (M_1) developed from treated seeds, for example, suffers from growth inhibition, may be partly sterile, and may lose many plants before flowering and seed set (Masuda *et al*. 2004; Ojiewo *et al*.

2005, 2006a). These M_1 plants are genetic chimeras and may carry the desired mutations only in a small sector. Mutations are often recessive, deleterious and lethal because favourable mutations are expected to be already incorporated into the genome of crop plants during the history of their evolution. It is estimated that at least one deleterious mutation per individual per generation occurs (Kondrashov 1988), controlling the adaptability and determining the fitness of the plant population. Fitness is an expression to indicate the relative probability of survival and rate of reproduction of a phenotype or genotype (Suzuki *et al*. 1989).

Mutation frequency is the observed (or estimated) number of mutations at a given mutagen dose per population of cells, organisms, gametes, plants or plant parts at a specific mutant generation (van Harten 1998). Mutation frequency can be calculated as a percentage of mutants from the radiated seeds (M_0), surviving seedlings in the first mutant generation (M_1) or mutated plants in the second mutant generation (M_2) analysed (Gaul 1960). The latter method is advantageous because variations in progeny size due to differences in survival rate or the size of the plant area that is mutated do not affect the final result as would be the case when the whole surviving progeny is considered (Gustafsson 1940; Blixt *et al*. 1963). Suzuki *et al.* (1989) defined mutation frequency as the frequency at which a specific kind of mutation (or mutant) is found in a population of cells or individuals. This definition disregards the number of mutations that gave rise to the desired mutant. However, within one cell, more than one independent mutational event can take place. But, on the other hand, not all genes are expressed in each cell at each moment. Moreover, many mutations after being induced may be repaired by various mechanisms. In *S. villosum*, the earliest observable mutants were chlorophyll deficiency mutants (**Box 1**; Guftafsson 1940) whose frequencies varied with the mutagen dose, and male-sterile mutant frequency was later observed to follow a similar distribution trend (Ojiewo *et al.* 2005, 2006a). We propose an integrated approach involving computation of general mutation frequency (GMF) based on the surviving population and specific population frequency (SMF) based on the total number of mutants.

As ample potent mutagens are available, selection of desirable mutants is more the bottleneck for mutation breeding. This is not so critical when the desired trait is easy to see as e.g. early flowering or altered plant architecture, but selection procedures limit the prospects of mutation breeding when more complex traits such yields are the objective. Optimizing mutation frequency is paramount and must be empirically determined: if it is too low, too many plants will be required to discover mutations in a target gene; if it is too high, viability and/or sterility is likely to be a problem, especially if undesired. In order to detect segregants with reasonable probability, the amount of seed material to be subjected to mutagenic treatment and the number of plants in the M_2 progeny is a critical consideration. The required number of M_2 plants is determined by the segregation ratio for a single recessive gene and the probability of occurrence of homozygous mutants. If pollen is treated, the expected mutant generation is 3:1 but if seed is treated, often more than one cell of an embryo contributes to the next generation, requiring an increase of the plant progeny size, given the same radiation dose. Traditionally, it has been common for mutation frequency to be estimated on the basis of phenotype, using screens for seedling lethality (survival rate), embryonic lethality (seed set), chlorophyll deficiency or single-copy gene phenotypes as a measure (Emery 1960; Prina and Favret 1983). We have used three replicates of 100 seeds (total 300 seeds) with some level of success in obtaining sufficient germination rate and survival frequency of tetraploid *S. villosum* (Ojiewo *et al*. 2005, 2006a). On the basis of overall plant development from germination, seedling survival, production of M_2 selfed-seed, GMF (total number of lines segregating observable mutants per M_2 progeny line) and SMF (frequency of male-sterile mutants per total number of mutants), we recommend that between 300~500 seeds should be used for determination of optimum mutagen doses for induction of mutagenesis in the polyploid series of African nightshade (**Table 1**).

The dose for ionising radiation, defined as the amount of energy absorbed per m^2 of irradiated matter at the point of interest (van Harten 1998) is determined by the intensity of the radiations and length of exposure. By varying mutagen dose, the frequency of induced mutations can be regulated and saturation can be readily achieved (Jander *et al*. 2003). The optimum radiation dose has been traditionally based on the LD_{50}; the dose at which 50% of the irradiated material die as a result of the treatment (Brock 1976). In mutation breeding practice with γ ray irradiation, a growth reduction of M_1 seedlings of 30-50% or a survival rate of 40-60% in comparison to the control plants is taken as a criterion for a promising radiation treatment (van Harten 1998). Assuming that all surviving plants are fertile, 50% lethality in M_1 generation of 300 seeds would lead to 150 M_1 plants from which to obtain M_2 seed. Generally, not all M_1 plants will be self-fertile. Assuming 50% self-fertility, the population would be further reduced to 75 plants. We used 16 plants per M_2 progeny line, but here we propose 20 plants giving a total of 1500 available for M_2 progeny screening, which may be too low. We propose therefore, that for induction of mutagenesis in the polyploid series of African nightshade, rather than the LD_{50} as a criterion for determining the optimum radiation dose, the dose at which the survival frequency initially declines be considered. Taking this critical threshold level to be 95%, and assuming 50% fertility rate, a total of 2850 M_2 plants will be available for screening. Some doses at which the survival rate initially decline with ion beam treatment are shown for various crops (**Table 2**). Depending on the ploidy level and the method of M_2 screening, the population size of the M_1 generation can be reduced or increased. Using complex modern molecular techniques, the population size may be much smaller. For example, in hexaploid and tetraploid wheat, only 1920 plants were required to identify 246 induced mutations in the waxy locus (granule bound starch synthase I). This exceptionally high mutation frequency was attributed to the polyploid wheat genome buffering against deleterious mutations, ensuring a high survival frequency and fertility rate (Slade *et al*. 2005).

Early researches on the effect of polyploidy on induced mutagenesis reported that mutagenesis is easier to induce in diploids than polyploids. In oats and wheat, only a few chlorophyll mutants could be observed in M_2 progeny (Stadler 1929). Allopolyploid cereal species were reported to tolerate chromosomal aberrations more than diploids (MacKey 1954). For a number of crops, polyploid species in most genera were more resistant to radiation than diploid counter-

Box 1 Types of chlorophyll mutants.

1. **Albino**: neither carotenoids nor chlorophyll is formed.
2. **Xantha**: Chlorophyll may or may not be produced but carotenoids dominate.
3. **Albovirids**: have different colours at the leaf base and leaf tip. These may further be subdivided into *alboxantha*, *viridoalbina* and *albovirids* (*sensu stricto*).
4. **Virids**: these have uniform yellowish green or light green colour already at the seedling stage. These can further be subdivided into:
   a) *Virescens*: light green gradually changing to dark green and mostly viable;
   b) *Chlorina*: are yellowish green and often viable;
   c) *Lutescens*: leaves wither and turn yellowish. This type is usually lethal;
   d) *Albescens*: looks more or less like lutescens but usually more extreme and lethal.
5. **Tigrina**: These have transverse brownish or yellow stripes due to transverse destruction of pigments.
6. **Striata**: These have longitudinal white or yellow stripes due to longitudinal destruction of pigments.
7. **Maculata**: These show spots of chlorophyll and/or carotenoid destruction distributed all over the leaf.

Table 1 Proposed mutation breeding scheme for African nightshade.

Season	Generation	Operation
1	M_0	• Subject about 300-500 seeds to mutagen treatment. The doses vary according to the mutagen type. • For γ rays, between 50~100Gy; for carbon-ion beam, between 20~50 Gy should be sufficient (Ojiewo *et al*. 2005, 2006a).
2	M_1	• Germinate the seeds and space-plant the seedlings in isolation to reduce chances of cross pollination. Unwanted out-crossing risk is however limited in African nightshade due to strong autogamy. • In case of dominant or maternally heritable mutations, these may be observable in M_1. However, since most induced mutations are recessive, there may be no observable off-types in this generation. • Harvest at least 20 self-fertile seeds from the surviving population for screening in the next generation or more of repeat screens may be necessary.
3	M_2	• Grow progeny rows of each plant and observe the plants from seedling stage through to maturity. The earliest observable mutants are chlorophyll deficiency mutants (See **Box 2**). • Identify rows showing desirable mutants and isolate the mutant plants on the basis of their morphology e.g. male-sterility. • If the objective involves quantitative traits, select normal, fertile and vigorous plants for such characters. • Harvest the seed of each selected plant separately.
4	M_3	• Grow individual plant progeny in rows. • Select and bulk if appropriate tests and observations show homogeneity of the desired trait in a given line. • If progeny rows show variation for the desired mutant, select the mutant and harvest individually. • Some traits may be linked to the desired trait and only expressed in M_3. For example a tomato mutant isolated in M_2 on the basis of broad leaf features was parthenocarpic in M_3 progeny (Masuda, unpublished).
5	M_4	• Conduct preliminary trials of the bulk or individual line progeny to establish stability of the trait and select the best line for further evaluation. • Reject segregating lines or those in doubt.
6-9	M_5-M_8	• Conduct multiseasonal or multilocational trials.
10		• Seed multiplication and release if the above trials show consistency and stability of the trait in question.

Table 2 Initial decline of survival rate with ion beam dosage.

Radiation type	Plant species	Dose (Gy)	Reference
220 MeV $^{12}C^{5+}$	Rice	10~20	Mizobuchi *et al*. 2001
	Spinach	20~30	Hata *et al*. 2006
	African nightshade	20~30	Ojiewo *et al*. 2006a
	Tomato	30~50	Masuda *et al*. 2004
	Eggplant	30~50	Mizobuchi *et al*. 2001
	Melon	80~100	Katai *et al*. 2001
	Arabidopsis	200	Tanaka *et al*. 1997
50 MeV $^{4}He^{2+}$	Rice	150~200	Mizobuchi *et al*. 2001
	Spinach	150~200	Hata *et al*. 2006
	Tomato	200~250	Masuda *et al*. 2004
	Eggplant	200-250	Mizobuchi *et al*. 2001

parts (Swaminathan 1965). In polyploids, the target gene loci for a trait subjected to mutagenic treatment is higher than for diploids. Consequently, the mutation frequency may be the same on a per locus basis but higher in diploids on a per trait basis. Conger *et al*. (1982) studied correlations between interphase nuclear volume (NV), the average meristematic interphase chromosome volume (ICV; NV divided by the number of chromosomes) and radiosensitivity at different ploidy levels and found that ICV has a more consistent correlation to radiosensitivity on the basis of plant lethality than NV. We summarise here from various recent studies some optimum γ-ray radiation doses based on ICVs of various plant species (**Table 3**). Within the same genus, section or species the NV is positively correlated with the ploidy level and with the target size of radiosensitivity. Based on this radiosensitivity in the various ploidy series of African nightshade is expected to vary. While lethality may remain the same due to gene redundancy in polyploids, male-sterility may be more difficult to induce in polyploids due to their higher buffering capacity which allows chromosomal aberrations in plant cells without affecting their viability. Although male-sterile mutants were easily induced in the tetraploid *S. villosum*, we have observed that chromosome doubling with colchicine restores fertility and floral structure in an abnormal floral organ mutant (Ojiewo *et al*. unpublished). Confirmatory tests that will strongly link polyploidy to fertility and organ structure restoration in this mutant are underway. Even if mutations other than male-sterility are desired, polyploids may carry such mutations and only transfer them to later sexual progeny. In addition, somatic mutations may remain hidden, resulting in relatively fewer mutations obtained.

In *Arabidopsis thaliana*, the doses required for lethality and sterility seemed to be different probably due to a difference in the number of target cells (Shikazono *et al*. 2003). In the case of lethality, some surviving cells in the meristem could rescue the plant from death. In contrast, in the case of sterility, a single cell directly affects the viability of the gamete. In order to inactivate the plant, all the cells having the potential to maintain meristematic function need to be killed, but in order to make a plant sterile, only a single cell needs to be inactivated, providing that no selection of cells takes place at the vegetative stage (Dellaert 1980). Induction of sterility using ion beams was a reflecion of rearrangements of the chromosomes, such as inversions, deletions, substitutions, duplications, insertions and reciprocal translocations (Redei and Koncz 1992; Shikazono *et al*. 2003). A similar value of relative biological effects (RBE) between sterility and chromosome aberrations in pollen mother cells has been reported after heavy ion irradiation (Mei *et al*. 1994).

MUTATION SPECTRUM IN AFRICAN NIGHTSHADE AND RELATIVE BIOLOGICAL EFFECTS OF IONIZING RADIATION

Biological effects after exposure of plants to mutagens may be somatic or genetic. Genetic effects are heritable because they are present in the germ line of the affected individual. Induced germline mutations have been used as a source of novel variation in both crop plants and experimental organisms since the 1920s. γ-irradiation has been used to induce several mutants of agronomic importance including male-sterility in tomato (Masuda *et al*. 1998, 1999) and African nightshade (Ojiewo *et al*. 2005). Their biological effect is based on the radiolysis of water to produce free radicals that damage different important compounds of plant cell (Kovács and Keresztes 2002). Studies on the formation of γ mutant plants demonstrated that irradiated *Vicia faba* (faba bean) was delayed by several days in the uptake of tritiated thymidine, indicating that entry into synthesis phase (S-phase) of the cell cycle was postponed by radiation treatment (Evans 1965). The arrest of seedling development induced by irradiation of plant seeds could probably be due to death of meristematic cells or an arrest of cell division. A comparison of the shoot apical meristems of arrested seedlings to that of non-arrested seedlings of *Arabidopsis* revealed a substantial difference in the ability of cells to progress through mitosis (Preuss and Britt 2003). None of the γ-irradiated mutant sections had a mitotic figure, indicating

Table 3 Optimum dose for seed irradiated with γ-ray.

Dose					
<50	**50~150**	**150~250**	**250~350**	**350~450**	**450<**
Bulb onion (29.4*) Fujimaki *et al*. 1992; Ukai 2003	Spinach (10.8) Hata *et al*. 2006	Tomato (7.4) Masuda *et al.* 1998	Water melon (?) Fujimaki *et al*. 1992; Ukai 2003	Pumpkin (6.7) Fujimaki *et al.* 1992	Carrot (5.8) Fujimaki *et al*. 1992; Ukai 2003
Welsh onion (29.5) Fujimaki *et al*. 1992; Ukai 2003	Garden pea (15.1) Fujimaki *et al*. 1992; Ukai 2003	Snap bean (8.7) Fujimaki *et al* 1992; Ukai 2003		Cucumber (6.5) Fujimaki *et al*. 1992; Ukai 2003	
	African nightshade (?) Ojiewo *et al*. 2005			Radish (5.2) Fujimaki *et al*. 1992; Ukai 2003	
				Melon (?) Masuda and Murakami 2003	

* Interphase chromosome volume.

that their shoot apical meristems were arrested at some point outside the mitosis phase (M-phase) of cell cycle. The full cycle of cell development is a fundamental necessity if the agronomic benefits attributed to mutation breeding are to be obtained without compromising already existing useful traits. Hence, optimum range of radiation doses at which the mutant trait of interest is transferred at high frequency to the next generation is a critical consideration. Studies on the ultrastructural effects of ionizing radiations on constituents of plant cells have also revealed anomalies including dissolution of the cell wall middle lamellae due to radiation damage of pectin (Kovács and Keresztes 2002).

The biological effects induced by heavy ion beams may be different from those induced by low LET radiation such as X- and γ-rays. Heavy energy ions can alter cell growth and induce various chromosomal aberrations including micronuclei, chromosome bridges, fragments and laggards (Wei *et al*. 2006). It has also been reported that the mutation frequencies by carbon ions are 8–33-fold higher than those of electrons and that ion beams can produce large DNA alterations such as inversion, translocation and large deletion as well as point mutation (Shikazono *et al*. 2001). Various studies on cell inactivation and genetic changes induced by ion beams as well as the relationship between LET and RBE have been performed. Ion particles show considerable RBE on lethality, tumorigenesis and pollen viability. Ion beams have been shown to be effective in inhibiting growth (Tanaka *et al*. 1997) and in inducing mutations (Mei *et al*. 1994; Nishimura *et al*. 1997; reviewed in Teixeira da Silva 2006) and chromosome aberrations (Vasilenko and Sidorenko 1995; Wei *et al*. 1995). In *Pisum sativum*, Vasilenko and Sidorenko (1995) reported that He ion beams with a LET of 0.95 keV/μm produced 6 times more micronuclei than did ^{60}Co γ-rays. In *Nicotiana tabacum*, Hase *et al*. (1999) reported that the frequencies of mitotic cells with chromosome aberrations, such as chromosome bridges, acentric fragments and lagging chromosomes in the root tip cells of the exposed seeds, increased linearly with increasing ion beam doses and that the relative ratios of the chromosome aberration types were significantly different between the ion-beam and the γ-ray regimes; chromosome fragments were more frequent in the former, and chromosome bridges in the latter. The effectiveness of high-LET heavy ion in inhibiting *Arabidopsis* (Hirono *et al*. 1970) and rice (Mei *et al*. 1994) seedling growth, reducing plant fertility, inducing chromosomal aberration and micronuclei in root tip cells and pollen mother cells of the first generation plants developed from exposed seeds, and inducing mutation in the second generation, were greater than that of low-LET X- or γ-rays. In *Arabidopsis*, novel mutants such as one with spotted accumulations of pigment in the testa (Tanaka *et al*. 1997), resistance to ultraviolet-B (Tanaka *et al*. 2002), flower with serrated petals and sepals named frl1 (Hase *et al*. 2000) and mutants that contain DNA rearrangements such as gl1-3, tt4 (C1), and ttg1-21 (Shikazono *et al*. 2001) have been isolated.

There are numerous comparative studies on the RBE of ion beam and γ-ray irradiation in ornamental plants (reviewed by Jain 2006), but similar reports in leafy vegetables and herbs are scanty. Some of the striking examples of novel mutants in ornamentals include specific flower colour mutants induced by ion-beam irradiation in chrysanthemum (Nagatomi *et al*. 1998) and carnation (Okamura *et al*. 2001), but not obtainable with γ-ray irradiation. Yamaguchi *et al*. (2003) reported a high frequency of mutation in rose even with doses of ion-beam that did not affect survival. The mutation spectrum included mutants with smaller or larger flower size, fewer or more petals and lighter or darker flower colour. In African nightshade we reported higher mutation frequencies of male-sterile mutants with defective pollen and anthers using carbon-ion beam (Ojiewo *et al*. 2006a) than those induced using γ-ray (Ojiewo *et al*. 2005). In addition, a novel temperature-sensitive male-sterile mutant with abnormal floral organs (T-5), whose flowers are "vegetative" under low temperature conditions from early-winter to mid-spring, stamenless under fluctuating temperature conditions in late-spring, indeterminate under high temperature conditions in summer and whose structure and fertility is partially restored, with berry- and seed-set in autumn, was induced with ion beams (Ojiewo *et al*. 2006a). Besides the mutation effect, it was also reported that the properties of some crops were changed significantly. Of 1411 radiation treatment derived mutant cultivars of various crops officially released by December 2000, about 5% (70), had novel properties not inducible by low LET ionizing radiation (reviewed by Maluszynski *et al*. 2000). In addition eight novel varieties of grain legumes with various properties not inducible with γ- or X-ray irradiation were induced by heavy ion mutagenesis (reviewed by Bhatia *et al*. 2001). Novel varieties with vigorous growth or higher yields have been reported in sugar beet or tomato after irradiating seeds with heavy ions (Liu *et al*. 2004). Through seed irradiation with N^+ ion beam, Wang *et al.* (2007) reported the isolation of a novel soybean mutant with multifoliate compound leaves as opposed to the wild type trifoliate compound leaves.

FLORAL BIOLOGY AND HYBRIDIZATION IN AFRICAN NIGHTSHADES

Typical wild-type African nightshade inflorescences (**Fig. 1A**; Edmonds and Chweya 1997) are cymose with simple or forked peduncles which are erect or reflexed at maturity peduncles holding up to 36 flowers. The flowers (**Fig. 1B**) are pentamerous hermaphrodites with the floral formula Ca^5 Co^5 A^5 G^1 (Ca: calyx; Co: corolla; A: androecium; G: gynoecium). The calyx is campanulate-stellate with broadly triangular to ovate-lanceolate sepal lobes, the mature sepals being reflexed or accrescent and externally pubescent. The corolla are usually <20 mm in diameter, white to purple, often with a conspicuous stellate to rotate basal star, the mature petals being recurved and externally pubescent. The filaments are fused for approximately half their length, joined to the corolla tube, and covered with uniseriate multicellular hairs. The anthers are connivent, oblong, and yellow to brown, dehiscing by oblong pores which often develop

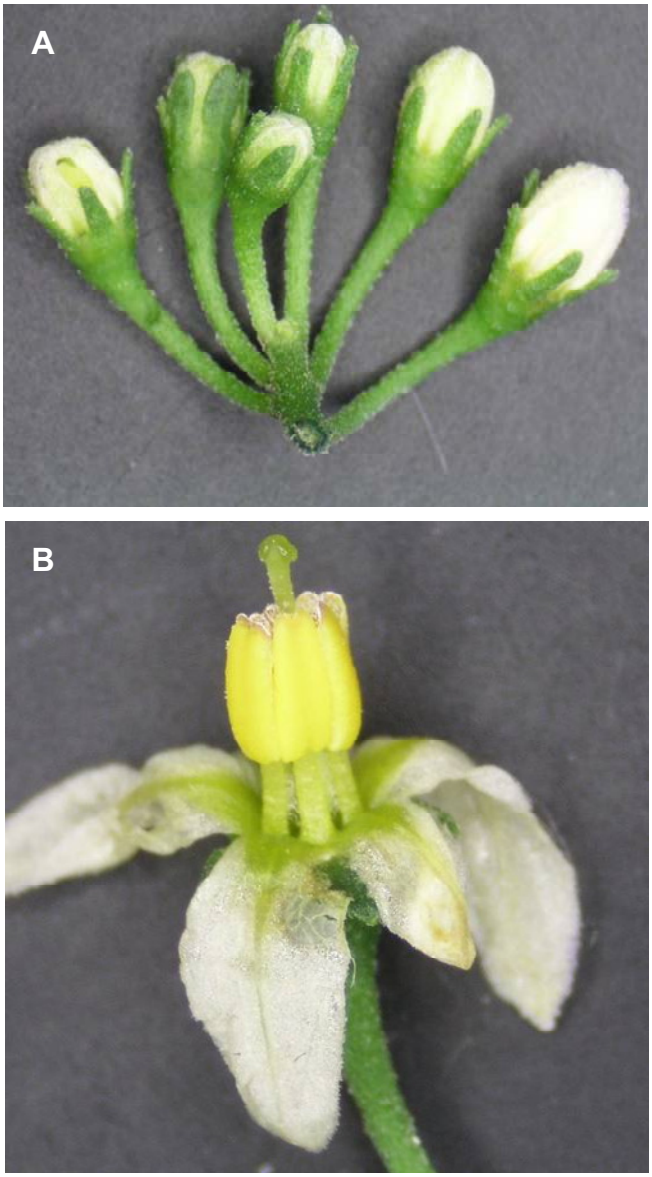

Fig. 1 Photograph of *Solanum villosum* flower showing a typical representation of African nightshade inflorescence buds and mature flower structure. The flower consists of campanulate-stellate calyx, with 5 triangular to ovate-lanceolate green leaf-like sepal lobes; white to purple corolla, a conspicuous basal star with 5 showy petals; 5 stamens with fused or semi-fused filaments and fused or semi-fused oblong yellow anthers; and single carpel with straight exserted style and capitate stigma.

into longitudinal slits. The pollen is spheroidal to subprolate in shape with a diameter of 17-40 µm. The styles are straight or geniculate, often exserted with globular capitate stigmas.

Solanum nigrum-related species are predominantly self-pollinating, but out- and cross-breeding can occur (Edmonds 1979). Natural hybridization is probably more widespread in the *Solanum* section *Solanum* than generally supposed but it is mostly followed by subsequent genetic breakdown in the F_1 and F_2 generations, thus limiting their existence (Edmonds and Chweya 1997). Cases of inter- and intra-specific hybridization have been reported, especially among the smaller-flowered diploids (Edmonds 1979; Edmonds and Chweya 1997). Natural hybrids have also been reported at higher ploidy levels, e.g. intra-specific hybrids of the hexaploid *S. nigrum* (Venkateswarlu and Rao 1972). In spite of variation in ploidy levels, natural hybridization has been observed between some *S. nigrum*-related species such as the hexaploid *S. scabrum* and the diploid *S. americanum* where accession distribution overlaps (Henderson 1974). Small bees, bumble bees and black syrphid flies are probably responsible for cross-pollination (Schippers 2000). Some species have flowers that, though visually white to pale purple, apparently have a hidden ultraviolet (UV) pattern which changes with the age of the flower; the bees are, therefore, visually sensitive to the flowers in both visual light and in the UV region of the spectrum. Though pollen is released only during the first 2 days following anthesis, which occurs at sunrise, the flowers remain on the plants for 10 days acting as a visual 'flag' to the pollinators (Buchmann *et al.* 1977).

For artificial hybridization experiments, emasculation of the flowers while still in the bud stage is usually necessary to prevent self-pollination as most species exhibit strong autogamy and will set fruit even when grown in insect-proof glasshouses. Plants selected as the maternal parents should be raised in insect-proof glasshouses, and the young buds emasculated l-3 days before pollination to allow full development and reflexion of the petals. The stigmas remain receptive for 3.5 days after the opening of the flower buds, and are thus unaffected by such early emasculation. As with natural hybridization, most taxa belonging to the section *Solanum* can be successfully hybridized artificially, at least initially, but morphological divergence is, generally, accompanied by genetic isolation due to genome disharmony and genic or chromosomal sterility with the crosses failing pre- or post-zygotically (Edmonds 1979). As a result, so far there are no improved cultivars or tangible variety development initiatives through conventional plant breeding techniques apart from selection of local variants or landraces in some of the regions in which these plants are utilized as food and/or medicinal plants (Edmonds and Chweya 1997). Mostly growing wild, semi-wild or as weedy forms, early and excess flowering coupled with prolific fruit production are adaptive features for survival and perpetuation of the species. However, competition resulting from this reproductive function reduces leaf productivity to extremely low levels. The consequence is poor yields that cannot compete effectively with high yielding exotic vegetables. Therefore, production of these vegetables has traditionally remained on kitchen-garden scales. Agronomic and cultural efforts to improve the yields of these vegetables have been numerous, but with little success. Notably, deflowering increased leaf yields by 40% (Mwafusi 1992). Deflowering is too cumbersome and labour intensive for small-scale farmers. Introduction of new varieties with improved and stable yields could be a more economically viable and technically feasible way forward. A promising strategy to achieve this is the induction and introduction of male-sterile lines with potentially high leaf yields and from which seed for propagation can be obtained by manipulating environmental conditions. The use of conventional mutagenesis would make the novel varieties potentially acceptable without resistance or complex legislations encountered by genetically modified organisms.

MALE-STERILITY

Stamens are the male reproductive organs of flowering plants. They consist of an anther, the site of pollen development, and in most species a stalk-like filament, containing a vascular strand which transmits water and nutrients to the anther. The filament provides support for the anthers and positions it to aid pollen dispersal. In African nightshade the filaments are extremely short while the anthers are usually larger (**Fig. 1B**; Edmonds and Chweya 1997). The anther has a single cell layer of epidermis tissue that surrounds four microsporangia. Each microsporangium is composed of an endothecial layer, two parietal layers, a tapetal layer and two cell layers of sporogenous tissue that give rise to the pollen grains. There are many recent reviews on anther specification in flowers (Theißen 2001; Lohmann and Weigel 2002; Becker and Theißen 2003; Jack 2004; Zahn *et al.* 2005). Floral organ specification in the *Solanum* section *Solanum* has also been reviewed elsewhere (Ojiewo *et al.* 2007a); therefore, the genetic regulation of stamen identity will not be discussed here.

Anther development initiates with the emergence of the stamen primordia in the third whorl of the floral meristem and concludes with the release of pollen grains at dehiscence. Within the stamen, primordia cell-specification and differentiation events give rise to mature anther cell types and generate the morphology of the anther and the filament. In many flowering plants, the anther has a four-lobed structure containing a stereotyped cell-type pattern that is repeated in each lobe (Goldberg *et al*. 1993). Histospecification, morphogenesis, and meiotic events constitute phase one of anther development. Phase two of anther development involves the functional programs that occur within differentiated anther cell types after tetrads have formed in the locules (Koltunow *et al*. 1990; Goldberg *et al*. 1993). The microspores differentiate into pollen grains, the filament elongates, the anther enlarges and expands, cell degeneration occurs, and the anther enters a dehiscence program that ends with flower opening (Goldberg *et al.* 1993). Dehiscence results in anther wall breakage at the stomium region located between the two locules of each anther half, or theca, and the release of pollen grains for subsequent pollination and fertilization. Anther developmental defects can generate male-sterile phenotypes that can be identified in sterility mutant screens.

The flowering plant life cycle alternates between a diploid sporophytic phase and a haploid gametophytic phase. Unlike in animals, where the products of meiosis differentiate directly into gametes, the meiotic products in plants (the spores) undergo mitotic divisions and develop into multicellular haploid structures, the gametophytes, which bear the gametes (Raven *et al*. 1999). The male gametophytes (microgametophytes) are pollen grains and the female gametophytes (or megagametophytes) are embryo sacs. Pollen is a highly specialized reproductive entity that performs a wide range of developmental functions, including cell specification and differentiation, cellular recognition, rapid polarized growth, chemotactic sensing, and fertilization (Lord and Russell 2002). Pollen development takes place in the male reproductive organs of the flower, the anthers and consists of several distinct stages that involve distinct changes in the sporogenous tissue (gametophytic) and in the tapetum and wall layers (sporophytic tissues) of the anther (Bedinger 1992). These stages can be largely divided into microsporogenesis and microgametogenesis (McCormick 1993). Microsporogenesis is the process of forming the microspore and encompasses the period from the formation of the sporogenous and tapetal initials through meiosis and the appearance of free microspores. Microgametogenesis covers the period from the first mitosis of the free microspores through the second mitosis that produces the gametes. A microspore mother cell undergoes meiosis to give rise to a tetrad of four microspores which are encased in a callose (β-1,3-glucan) wall. These uninucleate microspores are released upon the dissolution of the callose wall. Each uninucleate microspore undergoes an asymmetric mitotic division (the microspore mitosis) to give rise to two cells with distinct fates- the vegetative cell and the generative cell (**Fig. 2**). The larger vegetative cell is transcriptionally active (Mascarenhas 1990) and is thought to provide most of the proteins of the pollen grain. The vegetative cell forms the pollen tube during pollen germination but does not undergo any more cell divisions and therefore adopts a terminal cell fate. The generative cell is completely enclosed within the cytoplasm of the vegetative cell and is relatively transcriptionally quiescent (Mascarenhas 1990), but divides once more (the pollen mitosis) to produce two sperm cells (McCormick 1993).

Surrounding and interacting with the pollen mother cells is a transitory tissue, the tapetum, whose function is critical to the development of pollen (Mariani *et al*. 1990). It is a nutritive tissue and is known to provide compounds such as sporopollenin for incorporation into the pollen coat (Chapman 1987). During gametogenesis, the innermost cell layer of the anther, the tapetum, plays a crucial role for the release and nutrition of the microspores. Microspores are supplied with nutrients from the tapetum; therefore, mutations affecting tapetal development have led to abortion of microgametogenesis and male-sterility in maize (Cheng *et al*. 1979), *Arabidopsis* (Wilson *et al*. 2001) and petunia (Kapoor *et al*. 2002), among other plants. Tapetal cells secrete callase to release the meiotic tetrad from an enclosing callose wall. The exact timing and proper function of callase has been shown to be essential for pollen development. Precursors for the biosynthesis of the outer pollen wall (exine) are also supplied by tapetal cells, and even their remnants serve the pollen grain as tryphine or pollen kitt after degeneration (Bedinger 1992).

During the final stages of pollen maturation, the tapetal cell layers degenerate completely, with endothecium, epidermis, and connective tissue remaining functional, as anther dehiscence proceeds. The dehiscence program is temporally coordinated with the pollen differentiation process and triggers an ordered series of events within the anther culminating in anther burst and release of mature pollen (Goldberg *et al*. 1993). In some species, such as *Zea mays* and *A. thaliana*, the second mitotic division of pollen occurs within the anther before pollen release (anther dehiscence) and pollen tube germination (Regan and Moffatt 1990). However, in most *Solanaceae* plants such as tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*), this second mitosis does not occur until pollen tube germination (McCormick 1993; Liu and Palevitz 1996). The same could be the case in African nightshade (*Solanum* section *Solanum*), but no such studies have been conducted as yet. After germination the pollen tube grows into female tissues. The pollen tubes grow rapidly into the ovules, where the two sperms are delivered to the two female reproductive cells, resulting in double fertilization (Lord and Russell 2002). One sperm nucleus unites with the egg to form a diploid zygote, from which the embryo develops, and the other unites with two polar nuclei to form a triploid, primary endosperm nucleus. Upon double fertilization, the gametophytic phase ends and the sporophytic phase begins, thus completing the alteration of generations that is characteristic of the sexual

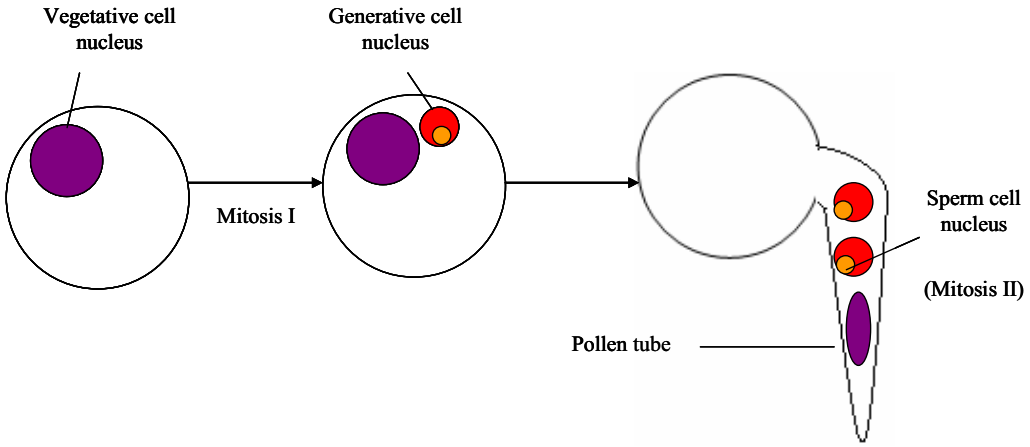

Fig. 2 A schematic representation of hypothetical pollen developmental stages in African nightshade. The vegetative cell forms the pollen tube during pollen germination but does not undergo any more cell divisions and therefore adopts a terminal cell fate. The generative cell divides twice to produce two sperm cells. The first mitosis takes place before anthesis but the second mitosis takes place after pollination and germination of the pollen tube.

Box 2 Classification of male-sterility.

a. Phenotypic

- **Structural male-sterility**: male sex organs are deformed, misformed, malformed or missing altogether. No microsporogenous tissue is developed or microsporogenesis fails to occur.
- **Sporogenous male-sterility**: stamens develop, but the sporogenous tissue either mis- or malformed. Pollen absent, non-functional or extremely rare due to premature microsporogenous cell abortion before, during, or after meiosis.
- **Functional male-sterility**: viable pollen form, but barrier prevents fertilization (anther indehiscence, no exine formation, inability of pollen to migrate to stigma or other factors that affect fertilization e.g. extended style, pollen is glued together so that it can't be released etc).

b. Genotypic

- **Genic male-sterility (GMS)**: Failure of pollen formation is because of one or more nuclear (dominant or mostly recessive) genes whose action is not affected by the cytoplasm type. Inheritance pattern and expression is Mendelian.
- **Cytoplasmic male-sterility (CMS)**: Based on the cytoplasmic genes only as a result of which the progeny of male-sterile plants is always sterile (non-Mendelian inheritance). The influence of the nucleus is minimal and the sterility is inherited maternally. Thus, irrespective of the nuclear genotype, plants with sterile cytoplasm (S-cytoplasm) are male-sterile and those with normal cytoplasm (N-cytoplasm) are male-fertile.
- **Cytoplasmic-genic male-sterility (CGMS)**: Male-sterility is due to the interaction between nuclear (*fr*) and cytoplasmic (mitochondrial) genes. CGMS plants have both Fr and fr nuclear genes as well as N and S cytoplasm. The S-cytoplasm contains cytoplasmic genes (*c*-genes), which in association with *fr* genes cause male-sterility.
- **Gene-environment induced male-sterility:** Male-sterility is due to the interaction between nuclear genes and environmental conditions such as temperature, photoperiod and soil conditions enzyme balance.

life cycle of plants.

Normal development of the stamen and pollen is essential for the successful completion of sexual reproduction in angiosperms. Abnormality at any stage in stamen and pollen development can result in male-sterility. Male-sterile mutants have been reported in several plant species (Kaul 1988). Male-sterility (*ms*) is defined as the failure of plants to produce functional anthers, pollen, or male gametes. The *ms* phenotypes may include complete absence or malformation of male reproductive organs (stamens) in bisexual flowers and no male flowers in dioecious plants, failure to develop normal microsporogenous tissue (anther), abnormal microsporogenesis (deformed or unviable pollen), abnormal pollen maturation (inability to germinate on compatible stigmata), nondehiscent anthers but viable pollen (sporophytic control) and barriers other than incompatibility preventing pollen from reaching ovule (**Box 2**; Frankel and Galun 1977; Kaul 1988). It can be controlled by nuclear or cytoplasmic genes, or both, that affect stamen and pollen development (Kaul 1988) and can be a result of gene mutation, inter- and intraspecific hybridisation, radiation, chemicals, genetic engineering or environmental factors (**Fig. 3**; Horner and Palmer 1995). In transgenic male-sterile mutants of *A. thaliana* anthers in transgenic plants became developmentally abnormal, with the pollen sacs completely missing at advanced stages of anther development (Sanders *et al*. 1999). The pollenless mutants had apparent meiotic defects and/or abnormalities in cell layers surrounding the locules. A male-sterile mutant exhibiting a pollen-less phenotype has been induced in *S. villosum* by seed irradiation with γ-rays and carbon-ion beams (Ojiewo *et al*. 2005).

In GMS mutants, the breakdown in microsporogenesis can occur at a number of pre- or post-meiotic stages. The abnormalities can involve aberrations in any one of the following stages: during the process of meiosis, in the formation of tetrads, during the release of tetrads (that is the dissolution of callose), at the vacuolated microspore stage, or at the mature or near-mature pollen stage (Kaul 1988). Apart from these cytological changes, male-sterility has also been associated with biochemical changes involving qualitative and quantitative changes in amino acids, proteins and carbohydrates in developing anthers. It has been reported that male-sterility could be induced through metabolic engineering of the carbohydrate supply (Goetz *et al*. 2001). Male-sterile mutants bearing mature pollen that are stainable normally with acetocarmine but, which stain positive for starch at and post anthesis have been isolated in tomato (Masuda *et al*. 1999) and *S. villosum* (Ojiewo *et al*. 2005). Fruit and seed set give the most exact estimation of fertility and pollen viability and fertilizing capacity in particular. Failure of this is, therefore, the initial step in isolating male-sterile mutants in a breeding scheme. Further studies to demonstrate cytochemically the composition and activity of enzymes, proteins, chromatin and carbohydrates could help establish the possible causes of pollen inviability and the resultant failure to fertilize.

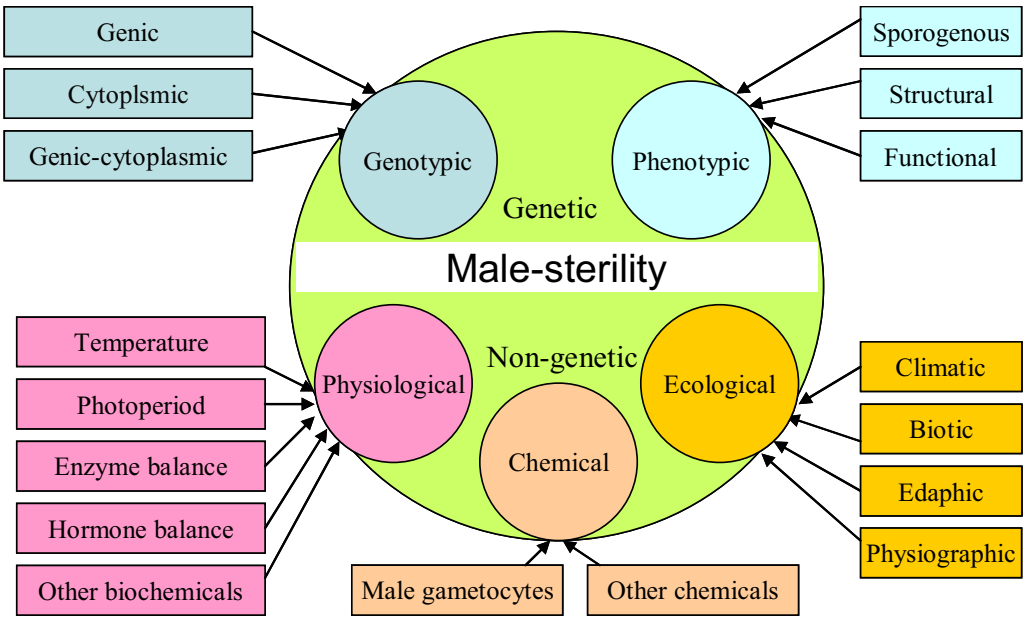

Fig. 3 Causes and types of male-sterility. Male-sterile mutations may be of genetic or non-genetic background and may arise either spontaneously or induced by chemical treatments, environmental manipulations or physiological and biochemical alterations. Classification of male-sterile mutants may be on phenotypic basis distinguishing the male sterile mutants on their expressional pattern or on genotypic basis relying on the genetic nature and inheritance pattern of a genotype.

DIRECT USE OF MALE-STERILE MUTANTS AS NOVEL VARIETIES

Hybridization for the production of hybrid varieties or for the introduction of genetic variation as a basis for selection, involves crossing female plants with male plants. In order to prevent self pollination, it is most convenient if the female parent is devoid of functional pollen. Male-sterility has been useful for interspecific hybridisation, performing backcrosses and production of F_1 hybrid seed in monoecious and hermaphrodite crops (Kaul 1988). This trait is potentially useful in interploidy breeding strategies discussed in details elsewhere (Ojiewo *et al*. 2007b). Here we focus on the direct use of male-sterile mutants as novel varieties with novel yield (or quality) attributes in African nightshade, an approach that has not been reported in any crop.

African nightshade plants produce very large amounts of small berries and seeds. A single *S. villosum* plant grown in a 15 cm diameter pot can produce close to 2,000 fruits each containing more than 60 seeds (Ojiewo *et al*. unpublished data). Among the African nightshades, as in most other herbaceous annual plants, vegetative growth is terminated by reproductive growth. As developing flowers and fruits are the major sinks for photosynthates and mineral nutrients, there is a decrease in the amounts available for the growth of other plant parts after anthesis (Salisbury and Ross 1992). Moreover, reproductive growth has been reported to suppress the growth of vegetative organs in a number of crops including cucumber (Marcelis 1992), tomato (Heuvelink 1997), banana (Eckstein *et al*. 1995), dandelion (Letchamo and Gosselin 1995) and chestnut (Famiani *et al*. 2000), sometimes necessitating the mobilization and redistribution of previously accumulated photosynthates and minerals (Gardner *et al*. 1985). In *Z. mays*, the source: sink imbalance during grain filling is accompanied by a sudden decrease in stover weight. The supply of assimilate by the sources and the demand of assimilate by the sinks is buffered by mobilization of assimilates temporarily stored in the stover (Martinez-Carrasco *et al*. 1993). This removal of dry matter from the stems during grain filling causes stem lodging in maize hybrids (Duvick 1992). One of the causes of leaf senescence at the whole-plant level is the source: sink imbalances that follow after anthesis and fruit set (Feller and Fischer 1994). Therefore, apart from inhibiting new leaf emergence and expansion of existing leaves, excess fruit load in African nightshade may also reduce leaf productivity by inducing early leaf senescence.

The partitioning of dry matter among various groups of organs depends on the number of organs per group and on their sink strength, i.e. their competitive ability to attract assimilates (Ho *et al*. 1989; Marcelis 1996). In tomato, 72% of the total above-ground dry matter production is distributed to the fruits (de Koning 1990, 1993). In gynodioecious *Sidalceae oregana*, male-sterile plants allocate more biomass, nitrogen, phosphorus and potassium to leaves than do hermaphrodite plants (Ashman 1994). *Plantago lanceolata* allocates 65% of the total flower dry mass to stamens and pollen (Poot 1997). Similarly, *Curcubita foetidissima* (Kohn 1989) and *Hebe subalpina* (Delph 1990) allocate large portion of the flower biomass to stamens and pollen. Pollen production is associated with relatively high respiratory costs. The tapetum contains a very high concentration of mitochondria showing that pollen production is high-energy demanding (Bedinger 1992). The high % N of the stamens relative to that of the other plant parts suggests a relatively high cost of protein construction (de Visser *et al.* 1992). It seems plausible to suggest that male-sterility or extremely reduced male-fertility has the potential to reduce the suppressing effect of the reproductive function over the vegetative function. Moreover, reducing the number of fruits per plant reduces the fraction of total biomass allocated to the fruits compared to the vegetative parts (Heuvelink 1997). African nightshade male-sterile mutants have been shown to have higher shoot tip fresh and dry weight than the wild type plants (Ojiewo *et al*. 2007c).

In many cases, the changed traits of officially released mutant varieties had synergistic effects on the cultivation of the crop, agronomic inputs, crop rotation and utilization (Maluszynski *et al*. 2000). For example, the short height genotypes in rice, wheat, barley and maize have contributed significantly to increasing grain yield because of their resistance to lodging and high planting density. The short height trait also allowed the use of relatively high doses of nitrogen. The early maturity of some mutants resulted in timely planting of the follow-up crop; for example early maturity of cotton in Pakistan allowed early planting of the wheat crop, resulting in higher wheat yield. The induction of thermo-sensitive genic male-sterile mutant in *japonica* rice (Maruyama *et al*. 1991), contributed significantly to the development of strategies for the production of hybrid rice varieties. Similar mutants have been induced by γ-rays in *indica* rice '26 Zhaizao' in China (Shen *et al*. 1993). Such mutants allow a 2-line hybrid seed production system instead of the usual 3-line system and show increased yield from heterosis. Due to the complexity of the yield trait, the direct economic advantage of the African nightshade male-sterile mutants may not be easily determined in absolute terms. Genes function in concert with other genes in a genome to alter the yield and quality of the end product.

PROPAGATION AND MAINTENANCE OF MALE-STERILE-MUTANTS

When male-sterility is controlled by a dominant gene only 50% of the progeny of a cross will be male-sterile. Male-sterility genes are however predominantly recessive and monogenic in nature, thus posing problems in the maintenance and propagation of male-sterile lines. Where male-sterility is inherited as a monogenic recessive condition, seed may be harvested after pollination with a homozygous (*MsMs*) or heterozygous (*Msms*) male-fertile plants. Pollination with *MsMs* results in a heterozygous (*Msms*) population which is phenotypically 100% fertile while pollination with *Msms* will segregate in the ratio of 50 % *Msms*: 50% *msms* (sterile). Male-fertiles in the segregating population have to be eliminated to leave a pure stand of male-sterile plants, but male-fertiles cannot be identified before flowering and it is undesirable to keep them that long. This practice is referred to as 3-line breeding system in hybrid seed production (**Fig. 4**) and has a number of limitations. First, it is uneconomical in terms of labour, inputs and space to maintain 50% of the population only to eliminate them at flowering. Second, it is difficult to prevent unwanted pollination of the male-sterile plants from this pollen source. These two limitations have been partially resolved for commercial production of canola hybrids (*Brassica napus*) in Canada by selective destruction of the male-fertile progeny by herbicide application (Mariani *et al*. 1989). Third, it is uneconomical also to propagate the maintainer for no other purpose than obtaining seeds from the male-sterile lines. Fourth, whether done manually or using chemicals, roguing of the undesirable male-fertile off-types is very expensive, labour intensive and time consuming. A number of strategies can be employed to circumvent these problems:

Environmental regulation of fertility

Environmental factors promoting male-fertility have been studied in several partially male-sterile plants. When conditions promoting fertility are very different from those required for sterility, maintenance of male-sterility may be easily achieved. Conditions promoting fertility can be used for inducing selfing and producing large amounts of male-sterile seed, while conditions promoting sterility would provide 100% male-sterile progeny. Male-sterility in rice *PGMS* (photoperiod-sensitive genic male-sterility) and *TGMS* (thermo-sensitive genic male-sterility) mutants is influenced by photoperiod and temperature (He *et al*. 1999; Wu *et al*. 2003). Rice *PGMS* lines are male-sterile under na-

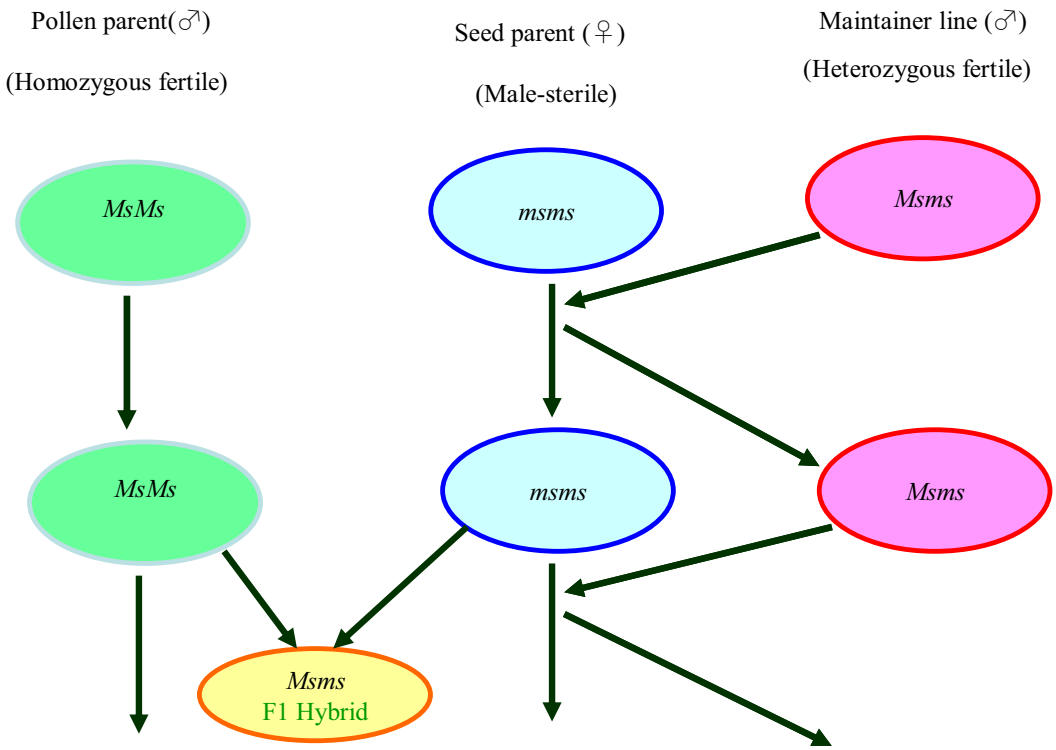

Fig. 4 Schematic representation of the conventional 3-line breeding system using genic-male sterile lines. The three-line breeding system requires, in addition to the seed and pollen parent, a third line, the maintainer line. The GMS line is maintained by backcrossing with the heterozygous maintainer line, producing a progeny that is 50% sterile and 50% fertile. The fertile plants have to be rogued.

tural long day and male-fertile under natural short day. Rice *TGMS* lines are sterile at high temperatures (>25°C) and fertile at lower temperatures. Viraktamath and Virmani (2001) reported that exposure to high temperature induced complete male-sterility in thermo-sensitive genic male-sterile lines while exposure of the young panicle to low temperature restored fertility in Annong S-1, a thermo-sensitive genic male-sterile line. Similar regulation of male-sterility by temperature has been reported in male-sterile lines of tomato (Sawhney 1983). Low temperatures were reported to restore male-fertility in stamenless-2 (*sl-2*) mutant of tomato (Singh and Sawhney 1998). Gómez *et al*. (1999) similarly reported that stamenless tomato mutant *sl* had its fertility restored in more than 15% of flowers that developed under low temperature. Mutant male-sterile tomato plants carrying *ms26* or *ms35* alleles can be rendered fertile by manipulating the day-time and night-time temperature, enabling the multiplication of these lines (Izhar and Firon 2001). Expression of male-sterility in mutants of Brussels sprouts (Nieuwhof 1968), barley (Sharma and Reinbergs 1976) and soybean (Stelly and Palmer 1980) was also reported to be temperature-controlled. In addition, temperature sensitive male-sterile mutants have been reported in model plants *Antirrhinum majus* (Zachgo *et al*. 1995) and *A. thaliana* (Sablowski and Meyerowitz 1998). Photoperiod-sensitive male-steriles have been reported in wheat (Jan and Qualset 1977), barley (Hocket and Ahokas 1979) and tomato (Sawhney 2004) and manipulation of fertility levels in these cases is possible by varying the photoperiods or growing in different locations or seasons. Fertility was induced in male-sterile mutants of *Daucus* (Michalik 1974) and *Sesamum* (Brar 1982) when grown in growth chambers under long photoperiods. Temperature and photoperiod-sensitive male-sterile mutants have potential for use in 2-line hybrid seed production systems (**Fig. 5**) and have been extensively tested and used in rice breeding. In a temperature dependent *S. villosum* male-sterile mutant, seed production can be achieved by temporal or spatial manipulation to synchronize flowering with temperatures of 20-25°C (day) and 15-20°C (night) (Ojiewo *et al*. 2006b). Within a very limited

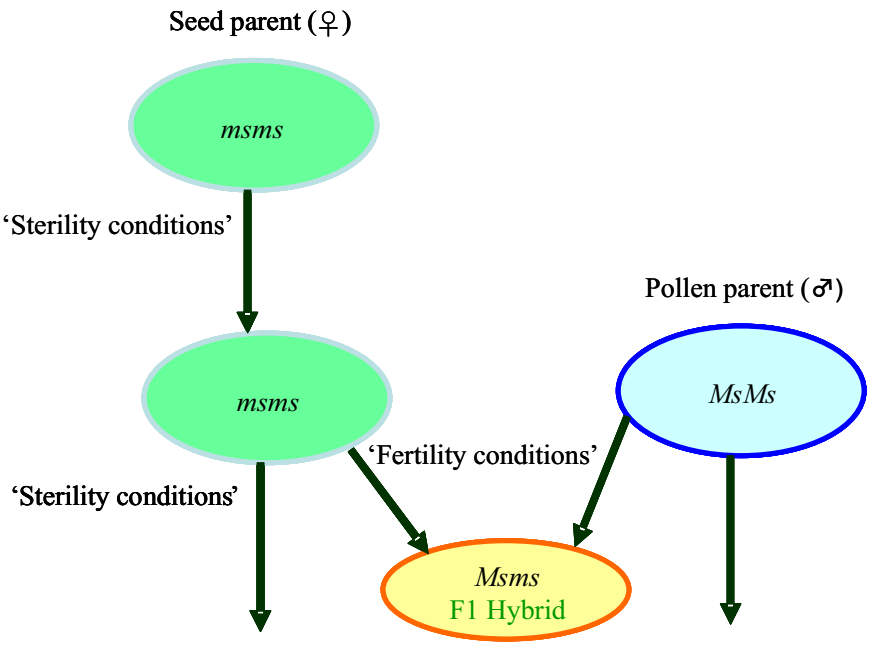

Fig. 5 Schematic representation of the 2-line breeding system achievable with conditional fertility restoration of the male-sterile seed parent. The system requires that the male-sterile parent be facultative so that it could be induced to self-pollinate when desired, thereby avoiding the maintenance of the male-sterile trait in the heterozygous condition. Theoretically, producing the male sterile parent under fertility conditions leads to 100% male-sterile progeny eliminating the need for a maintainer line or rouging of fertile plants.

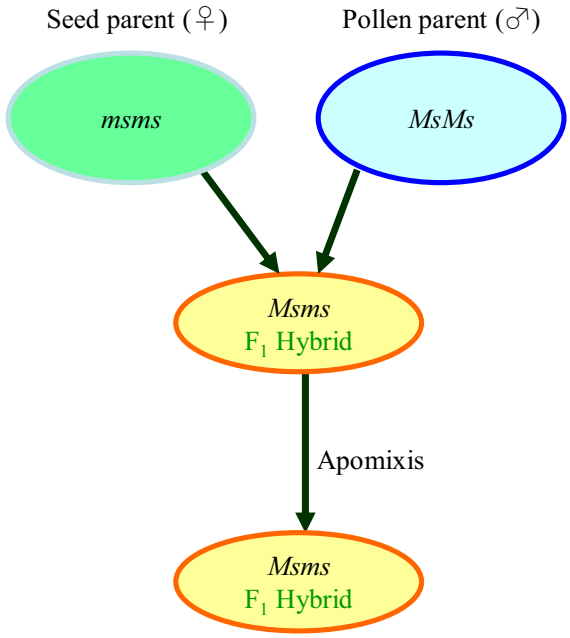

Fig. 6 Schematic representation of the 1-line breeding system making use of apomictic seed production of the F_1 progeny. In this case seed is formed asexually from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization, leading to embryo development. This phenomenon is not common in crop plants and most apomicts are facultative, and a proportion of the progeny may still result from sexual reproduction. In most cases, though embryo formation is asexual, endosperm formation requires fertilization.

season of late spring - early summer period when the flowers are mostly stamenless the mutant may be used as seed parent. However, as hybrid seed production is not the main goal in African nightshade male-sterility mutation project, 1-line breeding system is achievable where seed is obtained under fertility conditions and leaf production is done under sterility conditions. In hybrid seed production, 1-line breeding system is only conceivable where seed production of F_2 progeny from the F_1 cross is possible through apomixis (**Fig. 6**). However, apomixis is a rare phenomenon among cultivated crop species.

Partial male-sterility

Partial male-sterility is known in several crops. In some, the expression of male-sterility is influenced by environmental factors. In others, variable expression is seen between flowers or ears produced on the same plant. In other cases partial male-fertility is seen in all the flowers produced on a male-sterile plant. In these situations, selfing of the male-sterile plants to get all male-sterile progeny is possible. In wheat, a partial male-sterile with 5% selfing has found application in hybrid seed production programmes as the small amounts of selfed seed did not substantially affect heterosis of the hybrid seed. The female line was maintained by selfing the mutant (Gill and Anand 1970). In rice breeding, environmentally controlled male-fertility poses hybrid seed contamination risks as lines that are easily restorable often show partial residual fertility when grown under 'sterility' conditions and lines with tight male-sterility are often difficult to restore even when grown under 'restoring' conditions (He *et al*. 1999; Dong *et al*. 2000). Similar problems with residual fertility under 'sterility' conditions have also been reported in ps-2 tomato functional male-sterile lines (Kalloo 1993). Masuda *et al*. (2007) have developed a thermosensitive male-sterile tomato mutant that is partially fertile in autumn and sterile with 1.2% residual fertility in spring. The mutant has an additional advantage of leaf size marker, the selfed seed progeny (contaminant) being narrow-leaved. Where the goal is to use male-steriles as leafy vegetables, residual fertility can be taken advantage of for seed propagation, while the significantly reduced fruit and seed production is an asset in the regulation of vegetative-reproductive balance. A thermo-sensitive male-sterile *S. villosum* mutant with partial fertility restoration in autumn is generally sterile in spring but residual fertility has been observed under greenhouse conditions (Ojiewo *et al*. 2006b).

Chemical induction of fertility

If fertility can be restored temporarily in a male-sterile mutant through the use of synthetic organic or inorganic chemicals, multiplication can be achieved by chemical application that renders the plants fertile and the male-sterile can be selfed to obtain fertile seeds with sterile progeny. During the leaf crop production season, chemical application is not required and the plants remain sterile. In this case partial fertility restoration is sufficient to enable self-pollination of the male-sterile plant as about 10% viable pollen production in many autogamous crops leads to near perfect seed set. The choice of the chemical to be applied depends on the cause of fertility, especially its biochemical background. If a mutation causes male-sterility via lack of a metabolite needed for the production of functional pollen, fertility can be restored by exogenous applications of the missing metabolite. For example, fertility was restored to male-steriles by gibberellic acid in maize, tomato and barley (Nickerson 1960). Fertility in male-sterile mutants induced by branched-chain amino acid auxotrophy in the anther was restored with a mixture of the branched-chain amino acids (Dirks *et al*. 1994). Fertility in male-sterile tobacco (Taylor and Mo 1993) and petunia (Derksen *et al*. 1999) mutants induced by blocking the production of chalcone synthase in the flavonol biosynthesis pathway was restored by application of flavonols to the stigmas or to the pollen prior to fertilization. Fertility in male-sterile *Arabidopsis* mutants defective in jasmonic acid biosynthesis was restored by application of jasmonic acid (McConn and Browse 1996; Sanders *et al*. 2000). Fertility in male-sterile plants induced by overexpression of avidin resulting in reduced biotin levels was reversed by spraying the developing plants with biotin solution (Albertsen and Howard 1999). Lozano *et al*. (1998) proposed that abnormalities in tomato floral development caused by low temperatures may be attributable to changes in gibberellin biosynthesis and/or sensitivity. This was confirmed when treatment of apical shoot meristems of a stamenless tomato mutant plants grown at standard temperature with 0.5 mM GA_3 suppressed the homeotic transformation produced by the stamenless mutation in approximately 20% of the flowers (Gómez *et al*. 1999). We have tested the effect of GA_3 on the floral organ and fertility restoration in a homeotic mutant of *S. villosum* under summer and autumn conditions with the effect that GA_3 sprays enhanced the phenotype observed under low temperature i.e. large leaf-like organs in all whorls but did not improve fertility restoration (Ojiewo *et al*. unpublished data). Further biochemical studies to establish the pathways affected in male-sterile African nightshade mutants would provide useful guidelines as to which chemical to apply to induce fertility restoration for seed propagation.

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