INTRODUCTION
1. INTRODUCTION

The first ambitious objective, among the Millennium Development Goals, consists in the eradication of extreme poverty and food shortage by the 2015 target date. Nowadays, for fighting hunger and malnutrition using a sustainable and low-input farming system, plant breeding rather than agrochemistry and mechanization seems to be able to more efficiently increase food and feed production on less land and often in a more environment-friendly way. Plant breeding is focused on continuously increasing crop production to meet the needs of an ever growing world population, improving food quality to ensure a long and healthy life and address the problems of global warming and environment pollution, together with the challenges of developing novel sources of biofuels. Plant breeders use traditional techniques and biotechnology to create and use novel genetic variations, aimed at selecting new elite and suitable varieties, with improved traits to satisfy both farmers and consumers in terms of productivity, agricultural and quality performance, adaptability to marginal lands and regional environments, biotic and abiotic stress tolerance. Recent advances in biotechnology helps to enhance the efficiency and shorten the time required to reach the fixed purposes in a breeding program, as well as to address economic and ecological goals. Among these, haploid (H) and doubled haploid (DH) production through gametic embryogenesis has long been recognized as a valuable tool to help plant improvement.

Haploid plants are the sporophytes with a gametophytic chromosome number and doubled haploids (DH) are haploids that have undergone chromosome duplication (Germana, 2011; Kasha and Maluszynski, 2003). Thus in a diploid sporophytic (2n) species the haploids could also be called monoploid (x) as they have only one set of chromosomes. In polyploid species, the haploids (n) have more than one set of chromosomes and are polyhaploids. The production of haploids and doubled haploids through gametic embryogenesis allows a single-step development of complete
homozygous lines from heterozygous parents, shortening the time required to produce homozygous plants in comparison with the conventional breeding methods with carrying out several backcrosses and many generations of selfing which are time-consuming and labour intensive procedures (Morrison and Evans, 1987). Homozygous plants has had a significant impact on agricultural systems which are now a days have become an integral part of breeding program of many agronomically important crops (Germana, 2011).

Haploids, as a consequence of their unique genomic constitution, offer improved means of investigating many fundamental problems in genetics, cytogenetics and genomics. Mutations, gene-cytoplasmic and gene-environmental interactions can be more readily detected and studied without interference from factors such as heterozygosity. In addition, critical information regarding chromosomal homologies, chromosomal and genome evolution, chromosomal and gene dosage effects, the basis of meiotic pairing and other genetically controlled phenomena may be obtained from the study of haploids.

1.1 Methods of production of haploids and doubled haploids

Haploids occur spontaneously at a low frequency or they can be induced by several methods. A variety of methods used to obtain these haploids and DHs are in vivo modified pollination methods and in vitro culture of immature gametophytes. In vivo modified pollination method comprises chromosome elimination subsequent to wide hybridization, the “bulbosum” method developed by Kasha and Kao (1970) and pollination with irradiated pollen or pollen from a triploid plant. In vitro tissue culture methods consist of culturing the immature gametophytes resulting in gametic embryogenesis. Gametic embryogenesis, is named “‘gynogenesis’” when it initiates from a female gamete and “‘pollen embryogenesis or androgenesis’” when it starts from a male gamete (microspore or immature pollen grain) (Forster and Thomas, 2005). Gametic embryogenesis is one of the different routes of embryogenesis present in the plant kingdom, and it consists in the capacity of male (microspore or immature pollen grain) or female (egg cell) gametophytes
to irreversibly switch from their gametophytic pathway of development towards a sporophytic one. Each gametic cell possesses a unique genome where every gene is present as a single copy. Exploitation of this unique genetic unit and the totipotency of the plant cell is the basis of anther/pollen or ovary/ovule culture for the production of haploid plants. To date, almost 300 new superior varieties belonging to several families of the plant kingdom (particularly annual crops) have been produced by the above said means.

1.2 Spontaneous haploids

Natural haploids are the results of parthenocarpy (production of an embryo from an ovum without the participation of the male gamete) and apogamy (production of an embryo from a gametophytic cell other than the ovum). The male nuclei usually fertilize the polar nuclei, resulting in endosperm development and normal-appearing seed (Lacadena, 1974). Brassica, flax and sugar beet are the few species where spontaneous haploids have been reported (Jensen, 1986).

1.3 Wide hybridization and chromosome elimination (the “bulbosum” method)

Kasha and Kao (1970) first reported monoploid barley induction by crossing tetraploid Hordeum vulgare (2x = 4x = 28) as the female and tetraploid H. bulbosum (2x = 4x = 28) as the male. Pollen grains of H. bulbosum fertilize H. vulgare, but afterwards, chromosomes of H. bulbosum are eliminated from cells of developing embryo. It was demonstrated as embryo rescue, and growth of donor plants in controlled artificial conditions allow high frequencies of monoploid production (Jensen, 1986). Currently wheat x maize and wheat x pearl millet crosses are considered more efficient for haploid production in wheat (Matzk and Mahu, 1994). In tobacco also, chromosome elimination results in hybrids with somatic chromosome instability (Kasha, 1974). The chromosome elimination system for haploid production as applied in barley and wheat offers the advantage of producing large number of chromosomally stable haploids from any genotype.
1.4 *In vitro* haploid production

Over the last 20 years, one of the most intensive fields of research in plant biotechnology has been the widespread application of the *in vitro* haploid production methods based on artificial sporophytic development from gametes. *In vitro* haploid production methods involve the use of artificially induced stress (e.g. cold/heat, chemical factors) to influence the natural developmental and differentiation processes of male and female gametes. As a result of these stress factors, the development of the gametes is diverted from the gametophytic path of development to the sporophytic path, leading to the formation of androgenetic or gynogenetic embryos or morphogenic callus (Keller and Korzun, 1996b; Sopory and Munshi, 1996).

1.4.1 Androgenesis

The remarkable discovery that haploid plants and embryos can be produced by culturing anthers of *Datura* by Guha and Maheshwari (1964) bought renewed interest to haploidy. Anthers contain pollen and culturing the anther or isolated pollen induces these cells *in vitro* to undergo embryogenesis or haploid callus proliferation. These responses are induced by means of supply of various growth regulators to the culture medium. Pollen is haploid and the plants obtained from the pollen or microspores during culture are haploid as well. Haploids have been obtained *via in vitro* anther and microspore cultures in more than 240 species (Maheshwari et al., 1983). Since then, induction of haploids by androgenesis has become a successful strategy in various crop plants. Anther culture is often the method of choice for DH production in many crops because the simplicity of the approach allows large-scale anther culture establishment and application to a wide range of genotypes (Sopory and Munshi, 1996).

1.4.2 Gynogenesis by ovary/ovule culture

Gynogenesis is one of the methods for obtaining the haploids. Gynogenetic plants derive from the development of the female gamete (Chat et al., 2003). *In vitro* culture of
unpollinated ovaries and ovules represents an alternative for the production of haploid species for which anther culture has either given unsatisfactory results (yielding too many albinos) or has proven inefficient (Juokevieiene et al., 2005). Generally gynogenesis is the least favoured technique at the present time because of its low efficiency, but it has been applied to species that do not respond to more efficient methods (Forster et al., 2007a). Ovary and ovule cultures have been used for the production of haploids over a wide range of genera (Yang and Zhou, 1982; San Noeum and Gelebart, 1986; Keller and Korzun, 1996b; Lakshmi-Sita, 1997; Maluszynski et al., 2003). Gynogenesis may be the only efficient means of producing haploids in *Beta vulgaris* (Bornman, 1985), *Gerbera jamesonii* (Cappadocia et al., 1988; Cappadocia and Vieth, 1990), *Allium* spp. and other plant species (Keller and Korzun, 1996a) and is also useful for the comparison with paternally derived haploids.

1.4.3 Gynogenesis by means of irradiated pollen technique

Use of irradiated pollen for induction of *in situ* gynogenesis has been obtained by combining the use of irradiated pollen with *in vitro* culture of pollinated ovaries or immature seeds. In this method, the pollen is irradiated with varying doses of different radiations [X-ray, gamma (γ) rays] subsequently used to pollinate the female flower. Irradiation prevents the transmission of paternal nuclear genome to the egg cell and resulting gynogenesis is caused due to the absence of fertilization or by syngamy followed by preferential elimination of male chromosomes (Chat et al., 2003). This must be due to the damage caused by irradiation to the paternal chromosomes. Numerous attempts have been made to increase the frequency of haploids by treating the pollen, prior to pollination with various physical or chemical agents. For example, induction of female-derived haploid embryos following pollination with irradiated pollen has been used successfully employed in many species. Some of them are listed in Table 1.
1.5 Haploid identification

Haploids of higher plants can be distinguished from their diploid equivalents in many ways. They are smaller in appearance, partly because of their smaller cell size which is directly related to their ploidy level. Haploid status of the plant can be confirmed by various methods which include direct measurements of the chromosome number using conventional cytological techniques and measurements of the DNA content using flow cytometry (Bohanec et al., 2003), and indirect methods based on guard cell and plastid dimensions (Lee and Hecht, 1975; Quin and Rotino, 1995; Yuan et al., 2009). At a genetic level, although the detection of homozygosity in doubled haploids using isozyme-based techniques (Liu and Douches, 1993) is still practised (Bouvier et al., 2002; Grafe, 2003, Toppino et al., 2008), these have now largely been replaced by methods based on DNA markers (Verdoordt et al., 1998; Chani et al., 2000; Eimert et al., 2003; Belicuas et al., 2007; Diao et al., 2009). Cytological studies of cucurbits have lagged behind other crop plants primarily because their chromosomes are relatively small and stain poorly (Wang et al., 2007). Alternatively more simple, reliable, rapid and cost effective methods are available. Measurements widely used for the identification of ploidy levels are pollen grain diameter and stomatal length. The number of plastids in guard cells is also often used as a selection criterion in ploidy identification (Przywara et al., 1988; Kurtar et al., 2002, 2009).

1.6 Haploid exploitation

Haploids have been exploited in a wide range of theoretical and practical aspects of plant biology and genetics and some of them are:

1.6.1 Plant breeding

The most important use of haploids is based on the fact that marked improvements in the economics of plant breeding can be achieved via DH production as selection and other procedural efficiencies can be markedly improved by using true-breeding
(homozygous) progenies (Jauhar et al., 2009; Travadon et al., 2009; Wan et al., 2009). With stable DH production systems, homozygosity is achieved in one generation (Dunwell, 2010). Thus the breeder can eliminate the numerous cycles of inbreeding necessary to achieve practical levels of homozygosity by conventional methods. Indeed absolute homozygosity for all traits is not achievable by conventional breeding methods and this approach is impractical for self incompatible and male sterile plants and tree species. Consequently, an efficient DH technology would enable breeders to reduce the time and the cost of cultivar development relative to conventional breeding practices (Thomas et al., 2003; Forster and Thomas, 2005; Forster et al., 2007a).

1.6.2 Horticultural interest

Haploids may have intrinsic value because of their overall reduction in size compared with diploids. For example, the well-known cultivar of pelargonium (Li, 2005). In gentian, haploids may be produced by flower culture which may have future commercial value (Morgan et al., 2009).

1.6.3 Mutant isolation

Haploids are extremely useful for detection and isolation of recessive mutants which may not express in heterozygous diploid plants. Detection and isolation of such recessive mutants in the haploid state and rapid attainment of the mutated gene in a homozygous diploid state is special application of haploidy in higher plants. High photosynthetic activity selection in tobacco (Medrano and Primo-Millo, 1985), disease resistance in melons (Kuzuya et al., 2003), dwarf potato mutants deficient in gibberellin biosynthesis (Valkonen et al., 1999) are the successful examples. Similarly attempts have been made to produce useful haploid mutants in maize through anther culture (Ambrus et al., 2006; Darko et al., 2009), wheat (Bakos et al., 2008), Brassica species (Ferrie et al., 2008) and oilseed rape (Malik et al., 2008).
1.6.4 Transformation

Haploids also have a value in transformation programs. If haploids are transformed directly, then true-breeding diploid transgenics can be produced in one step following doubling of chromosomes. Various examples include those from rice (Chen et al., 2006), oilseed rape (Cegielska-Taras et al., 2008; Abdollahi et al., 2009), barley (Obert et al., 2008; Shim et al., 2009), poplar (Deutsch, 2004; Deutsch et al., 2004) and tobacco (Floss et al., 2009).

1.7 Cucumis melo L.

Melon (Cucumis melo L.; \(2n = 2x = 24\)) is a member of the Cucumis genus of the Cucurbitaceae family which comprises 30 species (Jefferey, 1980), which includes another important crop Cucumis sativus L. (cucumber). C. melo is the most variable species of the genus Cucumis and, even among vegetables, the great diversity of the fruit shape and size has always been recognized. There have been propositions to separate the genus Cucumis into two genera: Cucumis including the cucumber and Melo with the melon (Ashurmetov, 1995). Ambiguity regarding the origin of melon still exists with various authors putting forth their view towards the evolutionary aspects. Recent reports suggested wild progenitor of C. melo occurs in India (Sebastian et al., 2010). According to Wang et al., (2007) the centre of origin is very probably East Africa. Melon was first domesticated in Egypt around 3000 BC. Its cultivation was first spread to the Mediterranean, Middle East, and then to Asia. It was introduced to Europe, and then to the Americas from the Mediterranean. The worldwide melon production in the year 2010 is shown in the Fig. 1. China ranks the first place followed by Turkey and Iran. India ranks seventh position (FAOSTAT, 2012).

Wild melons with small, round or oval fruits, 20–50 gm in weight, are found commonly in Africa. Probably, originally, like many other cucurbitaceous members such as watermelon (Citrullus) and squash (Cucurbita), melon was domesticated for its seeds
because the fruit flesh was bitter. In Sudan, the seinat type is still cultivated for seeds. According to their use, melon cultivars can be divided into three main groups: those in which the fruits are not sweet at maturity, but are harvested at the immature stage and eaten raw, pickled or cooked, fruits that are harvested at maturity and are sweet, and fruits that are not edible but are used for their fragrance. Some rare cultivars of melon are cultivated for their leaves in southern Africa (Vorster and Jansen van Rensburg, 2004; Pech et al., 2007).

Melon is grown worldwide as a profitable crop. It is rich in nutrients (carbohydrate, organic acids, minerals and vitamins) and desired for its sweet aromatic flavour (Nunez-Palenius et al., 2008). Melon is among the 20 most important vegetable crops worldwide (FAOSTAT, 2012) mainly cultivated for the consumption of the fruits which can be harvested immature; in this case, the fruit is not sweet and can be eaten raw, cooked or pickled. In most cases, the fruit is harvested at maturity and high sugar content (mainly sucrose) is demanded. Fruit is mainly eaten raw; marginal uses are cubes canned in syrup, in “fruits confits”, candies, ice-creams, biscuits and also in cosmetics. Locally, seeds or leaves can also be consumed. Melon could have been domesticated for its seeds rich in proteins and lipids of good nutritional value (Pitrat, 2008).

1.7.1 Plant characters

Melon has usually a long main stem with several lateral branches. The repartition of the flowers at the different nodes is more regular in melon than in other cucurbits. At nodes of the main stem are located multi flowered inflorescences of male flowers. Flowers are quite large and hand pollination is easy to handle. Male flowers (in inflorescences) appear before female flowers at the nodes. The typical number of stamens is five but partial fusion leads generally to two large bilocular and one small unilocular stamens. The stamens open and release the pollen the day the corolla opens. The more the plant is ramified, the more female flowers are produced at the branches. Not every flower gives a
fruit as there is a strong competition between young fruits. Plants with 20-30 female flowers bear only 4-5 fruits. According to the temperature, a typical timetable is one month between sowing and the first male flowers, one month more to the female flowers and one to two months between pollination and fruit maturity depending on the fruit size and type.

The stigma is receptive one day before and one day after the corolla opens. It is usually more efficient to make pollinations in the morning, as in the afternoon nectar can wet the pollen. One successful pollination will produce 300 - 500 seeds (Pitrat, 2008). Melon is very susceptible to temperature and to light intensity and there is no all-year-round production of melon under glasshouses in temperate climate, northern or central Europe for instance (Pitrat, 2008).

Culinary melon (*Cucumis melo* L. var. *acidulus*) is endemic to tropical humid southern India and it is cultivated in Kerala, Karnataka, Andhra Pradesh and Tamil Nadu states. In local language, this melon is known as ‘Vellari/Mogekayi/Sambhar savate’. Fruits have very long shelf life and tender fruits are consumed as salad or used for ‘Sambhar’ preparation. ‘Sambhar’, a vegetable stew based on a broth with tamarind or ‘toor dal’; is a common dish in south India and Sri Lanka Tamil cuisines. Ripe fruits are eaten (Dhillon et al., 2007).

Snapmelon (*Cucumis melo* L. var. *momordica* (Roxb.) Duthie et Fuller) is native to India, where it is commonly known as ‘phut’ which means to split. They are also known as ‘ibbadalu/kekkare/milke hannu’. The fruits invariably crack at maturity. Immature fruits of snapmelon are cooked or pickled. The mature fruits with low sugar content are eaten raw and when ripe, the fruits invariably crack. Snapmelon germplasm has been found to be a very good source of disease and insect resistance (Dhillon et al., 2007). Powdery mildew, downy mildew, *Fusarium* wilt, zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV), cucurbit aphid borne yellow virus (CABYV) and *Aphis*
gossypii glover resistant snapmelon are being cultivated in various parts of India (Pitrat et al., 2000). A major goal of melon breeding is to develop resistance to the many fungal and viral diseases that affect this crop (Anagnostou et al., 2000).

Cultivated melon is diploid with 2n = 2x = 24 chromosomes. Until now, stable monosomic or trisomic plants have not been identified. More than 40 loci have been described in melon that confers resistance to viruses, fungi and insects (Pitrat, 2002). Most of these are dominant (Zym, Prv, Fom-1, Fom-2, Pm, Vat) and some of them display recessive inheritance, mostly those conferring resistance to viruses (nsv, cab-1 and cab-2). In general, regeneration from callus and other tissues of melon and cucurbits via somatic embryogenesis has not been very successful so far. This is due mainly to difficulties in the development of embryos and endo-polyploidy. In addition, the process is highly genotype dependent (Oridate et al., 1992; Pech et al., 2007).

1.7.2 Tissue culture studies in melon

Melon in vitro regeneration by organogenesis or somatic embryogenesis is not easy; one of the main problems is the great number of tetraploid plants which are regenerated. Shoot formation from cotyledons, hypocotyls, roots, or leaf explants has been obtained (Moreno et al., 1985; Kathal et al., 1994; Guis et al., 2000; Curuk et al., 2002). Somatic embryogenesis has also been successful (Oridate and Oosawa, 1986; Guis et al., 1997; Akasaka-Kennedy et al., 2004). Regeneration is genotype dependent and sexually transmissible; the genotype BU-21/3 has superior competence for regeneration by organogenesis (Molina and Nuez, 1996, 1997; Galperin et al., 2003a,b). Genetic transformations of melon with genes involved in disease resistance, salt tolerance, or long shelf-life have been successful (Fang and Grumet, 1990; Yoshioka et al., 1993; Clough and Hamm, 1995; Ayub et al., 1996; Bordas et al., 1997; Fuchs et al., 1997; Plages, 1997; Clendennen et al., 1999; Taler et al., 2004). For instance, transgenic Charentais-type melon plants with antisense ACC oxidase, the last enzyme in the biosynthetic pathway of
ethylene, have been extensively studied for shelf-life and ethylene-dependent or independent traits. Sugar and β-carotene accumulation are ethylene independent whereas fruit peduncle abscission, skin colour change, fruit flesh softening and emission of volatile compounds are ethylene dependent (Pitrat, 2008).

Efficient *in vitro* regeneration protocols are essential for successful and reproducible genetic transformation. In spite of number of protocols for the regeneration of melon *via* organogenesis, embryogenesis and from protoplasts, melon is still considered as being difficult to regenerate and there is a large dependence on the genotype for the capacity to regenerate (Pech et al., 2007).

**1.7.3 Haploidization and doubled haploids in melon**

There has been no major innovation in the production of haploid plants or in somatic hybridization in melon since the last review on melon biotechnology (Guis et al., 1998). Androgenesis in melon has not been reported till date. Similarly, culture of ovary has been limited only with very little success in production of haploids and double haploids in melon which is highly genotype dependent and cumbersome (Ficcadenti et al., 1999; Malik et al., 2011). No reports on ovule culture are also available. Induction of *in situ* haploid parthenogenesis by irradiated pollen is still the method of choice which has been tried by various authors (Sauton and Dumas de Vaulx, 1987; Lotfi et al., 2003; Lim and Earle, 2008, 2009). Again, the success rate is not so impressive. Some improvements have been proposed. A pre-culture of seeds in liquid medium before placing the embryos on a semi-solid culture medium significantly improved the number of haploid plants (Lotfi et al., 2003). Immersion of *in vitro* cuttings into a colchicine solution has been proposed by Sauton (1988) in order to double the chromosome number so as to obtain doubled-haploid plants. An alternative method, by immersing shoot tips of haploid plants grown under glasshouse conditions, is more efficient (Yetisir and Sari, 2003). The different steps in the procedure, i.e., the frequency of haploid plant production and the efficiency of
doubling the chromosome number, are genotype dependent, but there has been no study on their genetic control.

1.7.4 Breeding for disease and pest resistance

Various devastating diseases cause great losses to melon crops around the world. Among the important fungal diseases are *Fusarium* wilt, powdery mildew, downy mildew, *Alternaria* leaf blight and gummy stem blight. Various viral disease that are reported are from cucumber mosaic virus (CMV), papaya ringspot virus (PRSV), watermelon mosaic virus 2 (WMV2), zucchini yellow mosaic virus (ZYMV), cucurbit aphid-borne yellow virus (CABYV), squash mosaic virus (SqMV) and watermelon chlorotic stunt virus (WCSV). Similarly few insect species are known to infest melons such as white fly, aphids, leaf miner, beetles and fruit fly. Development of disease-resistant cultivars is one of the main objectives of melon breeding programs. Traditionally, the pedigree and backcross methods have been used for this purpose, but they require six to eight generations to obtain pure lines from a heterozygous source as in tobacco (Rufty et al., 1987).

Sources of resistance to pests and diseases are mainly found in accessions from India and Far-East belonging to the *acidulus*, *momordica*, *connomon* and *makuwa* varieties. Some accessions are particularly interesting as they cumulate resistance to several diseases. For instance PI 414723 from India is resistant to *Fusarium* wilt, powdery mildew, *A. gossypii*, ZYMV, PRSV, and CABYV; MR-1 from India is resistant to *Fusarium* wilt, powdery and downy mildews, and *Alternaria*; PI 161375 from Korea is resistant to CMV, *A. gossypii*, MNSV, and *Fusarium* wilt. Other geographical origins could also be interesting, for instance TGR 1551 from Zimbabwe which is resistant to powdery mildew, CYSDV, *A. gossypii* and WMV. All these accessions are cultivated and even if wild melons could be resistant to some disease, no resistance has been found only
in non cultivated melons. This could also be due to the fact that wild melons are underrepresented in collections (Pitrat, 2008).

Problems like seasonality, viral, bacterial and fungal diseases and insect pests have contributed to significant reduction in overall yields in culinary melon and snapmelons which are cultivated extensively in southern India. Progress in overcoming these problems by conventional breeding has been slow. There is scope for improvement of these two species via haploid breeding. This has led to more emphasis on the use of in vitro techniques especially gynogenesis for genetic improvement in them.

With these views, the objectives of the present study were:

1. To test the effect of various growth regulators individually on induction of gynogenesis in melon by means of ovule and ovary culture.
2. To test the effect of various growth regulators in combination on induction of gynogenesis in melon by means of ovule and ovary culture.
3. To test the effect of various doses of γ-irradiation on induction of gynogenesis in melon cultivars.
4. To test the effect of colchicine on induction of doubled haploids (2n) from haploid (n) plants of melon.
5. To acclimatize the regenerated haploid and doubled haploid plants.
6. To study the developmental aspects of the haploid embryo induction after pollination of female flowers with γ-irradiated pollen.
Table 1. Induction of gynogenesis by means of irradiated pollen technique in some plant species.

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<tr>
<th>Species</th>
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<tr>
<td>Barley</td>
<td>Powell et al., 1983</td>
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<td>Blackberry</td>
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<td>Cabbage</td>
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<td>Carrot</td>
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<td>Kiwifruit</td>
<td>Pandey et al., 1990; Chalak and Legave, 1997</td>
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<td>Melon</td>
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<td>Wheat</td>
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Fig. 1. Worldwide production of melon in 2010 (FAOSTAT, 2012).