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The potential of transgenic chickpeas for pest control and possible effects on non-target arthropods

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Abstract

Chickpea, *Cicer arietinum*, is the third most important grain legume crop in the world, with India being the largest producer. Insect pests are a major constraint to chickpea production. In India, the legume pod borer *Helicoverpa armigera* is the major insect pest of chickpeas. However, sap-sucking insects that act as vectors for viral diseases and bruchid beetles in storage are also considered important pests. Here we give an overview over the different management options to control these pests. There is a growing interest in the genetic modification of crops to enhance their resistance against insect pests. Here we present the state-of-the-art of chickpea transformation and give an overview on the available insecticidal genes that could be deployed to increase insect resistance in chickpea. Prior to commercialization, transgenic crops have to be assessed for their effects on the environment including the possible impact on non-target arthropods, many of which are important for biological pest control. Therefore, the arthropod–food web in the Indian chickpea system is described. Possible routes through which entomophagous insects could be exposed to insecticidal proteins expressed by genetically modified chickpeas are discussed, and species that could be selected for pre-release risk assessment are recommended.

Keywords: *Aphis craccivora*; *Callosobruchus* spp.; *Cicer arietinum*; Genetic engineering; *Helicoverpa armigera*; Insect resistance; Pest management; Risk assessment

1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume crop in the world after dry beans and peas (FAO, 2003). While this pulse crop is an important source of dietary protein for human consumption, it is also important for the management of soil fertility due to its nitrogen-fixing ability (Maiti, 2001). The chickpea probably originated in an area of south-eastern Turkey and adjoining Syria, but is now cultivated throughout the semi-arid regions of the world (Jodha and Subba Rao, 1987). Chickpea has enormous prospects especially under marginal land and water resource situations. Chickpeas are often divided into two major groups (Muehlbauer and Singh, 1987). The 'desi' types produce small, angular seeds that are variously pigmented and are grown principally on the Indian subcontinent and in East Africa. The 'kabuli' types have relatively large, round seeds of white or pale cream colour and are predominantly grown in the Mediterranean region and in Central and South America.

In 2002, the worldwide chickpea area harvested was 10.7 million hectares and total grain production was 8.2 million tonnes. India is the largest producer and consumer of chickpea in the world, and accounts for more than 60% of the area harvested and of the total worldwide production (FAO, 2003). Over the past 40 years, considerable progress has been made in chickpea production. While the grain yield in farmer's fields in India has increased from about 0.55 t ha^{-1} in the early 1960s to an average of 0.8 t ha^{-1}, productivity has stayed stable between 4 to 6 million tonnes which is primarily due to a decrease in the area harvested (FAO, 2003).

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Despite this progress, there is still a large gap in grain yields between the average productivity on farmer's fields and the maximum yield potential of up to 6 t ha^{-1} recorded in field trials conducted in the Mediterranean region (Singh, 1987). While low inputs and sub-optimal crop management accounts significantly for this gap, diseases and insect pests are also major constraints to chickpea production. The enhancement of insect and disease resistance in chickpea can increase its yield potential by as much as three times (ICRISAT, 1992). However, due to the lack of sources of resistance to these constraints in the available germplasm, the success rates of genetic enhancement of the chickpea by conventional plant breeding has been modest. Possibilities for further breakthroughs will therefore largely depend on alternate sources of resistance from wild species or through the use of modern tools in biotechnology.

Genetic transformation to enhance crop resistance or tolerance to biotic constraints is regarded as having good potential to achieve more sustainable food production in less-developed areas of the world such as the semi-arid tropics where agrochemicals are frequently inaccessible to farmers (Sharma and Ortiz, 2000; Sharma et al., 2001). Considering the potential impact that biotechnology can have on the livelihoods of resource-poor farmers, India is investing an increasing amount of resources into plant biotechnology (Paarlberg, 2001; Raghuram, 2002; Sharma et al., 2003a). The interest in genetically modified (GM) plants is likely to grow after the commercial release of transgenic cotton in 2002 that expresses the insecticidal *cry1Ac* gene of the soil bacterium *Bacillus thuringiensis* (*Bt*) (James, 2002), and the apparent success in controlling the pod borer *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Quaim and Zilberman, 2003). However, prior to deployment, each crop and transgene combination needs a close examination for its potential to benefit the poor (Atkinson et al., 2001) and possible environmental effects (Dale et al., 2002; Conner et al., 2003). Here we provide an overview of the most important insect pests of chickpea in India and their management as well as the potential to control these pests using GM chickpeas. We further present information on the beneficial arthropods that are active in the chickpea crop and suggest species that could be tested as part of a regulatory risk assessment.

2. Insect pests of chickpea and their management

Even though a number of insect herbivores have been reported to be associated with chickpea (Van Emden et al., 1988; CPC, 2001), only three groups, i.e. the legume pod borer *H. armigera*, sap-sucking pests [especially *Aphis craccivora* Koch (Hemiptera: Aphididae)] and bruchid beetles belonging to the genus *Callosobruchus* [*C. chinensis* Linnaeus, *C. maculates* Fabricius, *C. analis* Fabricius] cause major economic losses in India (Reed et al., 1987). One reason for the paucity of herbivores on chickpea is the dense layer of non-glandular and glandular trichomes, which cover the surface of all green plant parts, and the highly acidic exudate excreted by the glandular trichomes (Cubero, 1987).

2.1. Helicoverpa armigera

The legume pod borer is one of the most serious and widespread pests in the Old World. Its serious pest status has mainly been attributed to the high fecundity, extensive polyphagy, strong dispersal ability, and a facultative diapause. The larval preference for feeding on plant parts rich in nitrogen such as reproductive structures and growing tips results in extensive crop losses (Fitt, 1989). In chickpea, *H. armigera* is the dominant field pest and yield losses of up to 40% have been reported from farmer's fields in India (Reed et al., 1987; Srivastava and Srivastava, 1990). Worldwide losses due to *H. armigera* in chickpea have been estimated at over US$ 330 million annually (ICRISAT, 1992).

While a 1977–82 survey revealed that less than 10% of farmers used pesticide to control *H. armigera* in chickpea (Reed et al., 1987), the shift from subsistence to commercial production and the resulting substantial rise in the price of the crop has provided farmers with the opportunity to consider pest management options which formerly would have been uneconomic, leading to an increased use of pesticides (Shanower et al., 1998). A wide variety of insecticides have been used to control *H. armigera*, and in many areas, several applications are needed to contain this pest (Reed et al., 1987; Sachan, 1992). Intensive insecticide application to control *H. armigera* on various crops (especially cotton) has resulted in the development of resistance to the major classes of insecticides such as chlorinated hydrocarbons, organophosphates, synthetic pyrethroids and carbamates (Armes et al., 1996). This has resulted in control failures and a lack of confidence in chemical control among the farming communities (Raynolds and Armes, 1994).

Since 1976, more than 14,000 chickpea germplasm accessions and breeding lines have been screened for resistance to *H. armigera* at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) under open-field, pesticide-free conditions. Several genotypes with low to moderate levels of resistance were identified (Lateef and Sachan, 1990). However, while the resistant lines suffered less pod damage, they often produced lower yields than the controls, which was partly due to the relatively small seed size of some of the

resistant lines (Shanower et al., 1998). In addition, most of the resistant/tolerant lines were found to be susceptible to diseases, particularly to Fusarium wilt and Ascochyta blight (Lateef and Sachan, 1990). So far, few varieties with enhanced levels of resistance to *H. armigera* have been released for cultivation by the farmers (Sharma et al., 2003b). In recent years, much has been learned about the mechanisms of *H. armigera* resistance in chickpea. Ovipositional antixenosis is one of the components of resistance (Lateef, 1985; Cowgill and Lateef, 1996). In addition, inhibition of larval growth by malic acid and oxalic acid contained in the trichome exudates (antibiosis) appears to be an important mechanism (Cowgill and Lateef, 1996; Yoshida et al., 1995). Other antibiosis factors include *Helicoverpa* gut protease inhibitor activity in developing chickpea seeds (Patankar et al., 1999) and phenyl ammonialyase activity in leaves (Bhatnagar et al., 2000).

While a number of parasitoids and predators have been reported to attack *H. armigera* in India, their presence and impact in chickpea is generally low (Romeis and Shanower, 1996). The most efficient species is the parasitic wasp *Campoletis chlorideae* Uchida (Hymenoptera: Ichneumonidae), for which parasitism levels of up to 30% of young larvae have been reported (Romeis and Shanower, 1996). The low activity of natural enemies in chickpea is most probably due to the dense trichome layer on the plant surface and the acid exudates (Jalali et al., 1988; Murray and Rynne, 1994; Romeis et al., 1999).

The use of microbial pathogens including *Helicoverpa* nucleopolyhedrovirus (HaNPV), entomophatogenic fungi, nematodes and biopesticides such as *Bt*-products and neem seed kernel extract have shown some potential to control *H. armigera* (Saxena and Ahmed, 1997; Shanower et al., 1998; Lingappa and Hegde, 2001). In particular, HaNPV has been demonstrated to be a viable option to control *H. armigera* in chickpea as it can be as effective as the chemical pesticides (Rabindra et al., 1992; Cowgill and Bhagwat, 1996; Cherry et al., 2000). However, there are still two major obstacles for widespread adoption of this technology. The first is the high production costs that make the viral treatments uncompetitive compared with the synthetic insecticides, and the second is the lack of an effective product quality control system (Jenkins and Grzywacz, 2000; Cherry et al., 2000).

Cultural control options such as manipulation of plant spacing, time of sowing, intercropping and soil operations such as ploughing have also been shown to have some potential to reduce the damage caused by *H. armigera* (Reed et al., 1987; Shanower et al., 1998).

2.2. Sap-sucking pests

Sap-sucking pests infesting chickpeas reach pest status mainly due to the fact that they act as virus vectors. Aphids, especially *A. craccivora*, are known to transmit a large number of viral diseases in chickpea (Kaiser et al., 1990). The most important is a strain of the bean leaf roll luteovirus, the main cause of chickpea stunt, which is transmitted in a persistent manner by *A. craccivora* (Brunt et al., 1996). Another chickpea disease is caused by the chickpea chlorotic dwarf virus (Horn et al., 1995), a tentative mastrevirus (Fauquet and Stanley, 2003). This virus is transmitted in a persistent, non-propagative and circulative manner by the leafhopper *Orosius orientalis* (Matsumura) (Hemiptera: Cicadellidae) (Horn et al., 1994; Brunt et al., 1996).

Aphids are generally not controlled in the chickpea crop. While pesticides have been reported to be effective against *A. craccivora* (Sharma et al., 1991), their application is expected to be of limited value since the aphids would still transmit the virus before dying, therefore preventing only secondary virus spread (Reed et al., 1987). In addition, *A. craccivora* has already developed some levels of resistance to a number of common insecticides (Dhingra, 1994). Little is known on possible mechanisms of resistance in chickpea to aphid attack. Weigand and Tahhan (1990) have reported that a lower pH in leaf washings correlates with a smaller number of aphids found on the plant.

2.3. Bruchids

Damage in chickpea storage in India caused by *Callosobruchus* spp. varies among geographical regions with reported average infestation levels of up to 13% (Mookherjee et al., 1970; Dias and Yadav, 1988).

Even though chemical pesticides have the potential to give good protection against bruchid attack (Rahman and Yadav, 1987; Yadav and Singh, 1994; Lal and Dikshit, 2001), their adoption in chickpea storage appears to be small (Srinivasu and Naik, 2002). Extensive screening of seeds of kabuli type chickpeas against bruchids have not revealed any acceptable level of resistance (Weigand and Pimbert, 1993). In contrast, desi type seeds with enhanced levels of resistance have been reported (Raina, 1971; Schalk et al., 1973; Weigand and Tahhan, 1990). Unfortunately, resistant lines usually produce relatively small seeds with a rough seed coat which is unacceptable to consumers (Reed et al., 1987). One way of reducing the oviposition by bruchid beetles is to store split chickpea seeds as used for dal preparation (Reed et al., 1987). Biological control of bruchids has not really been exploited in India, despite the use of different plant products (Boeke et al., 2001). A comprehensive review of biological control of bruchids in the tropics has been published by Van Huis (1991).

3. Potential of transgenic insect-resistant chickpeas

3.1. Genetic transformation of chickpea: current status

Non-sexual DNA transfer techniques make possible the manipulations that are outside the repertoire of breeding and cell fusion techniques (Sharma and Ortiz, 2000). Genes can be accessed from exotic sources (plant, animal, bacterial, viral) and introduced in the crop of interest. However, the lack of availability of efficient transformation methods to introduce foreign genes can be a substantial barrier to the application of recombinant DNA methods in some crops including legumes such as chickpea. A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies. Several regeneration protocols involving somatic embryogenesis (Rao and Chopra, 1989; Barna and Wakhlu, 1993; Sagare et al., 1993; Dineshkumar et al., 1994; Suhasini et al., 1994) and shoot organogenesis (Shri and Davis, 1992; Barna and Wakhlu, 1994) in chickpea have been reported from diverse explants in both mature and immature tissues with varying success rates (Sonia et al., 2004). However, the recovery frequency of regenerated plants and their transfer to the glasshouse has been very low, which has limited the progress in genetic transformation of chickpea. More recently, an efficient and reproducible protocol for the regeneration of shoots at high frequency has been developed at ICRISAT that uses explants derived from the axillary meristems of cotyledonary nodes of in vitro-germinated seedlings of chickpea (Jayanand et al., 2003). This includes effective rooting of the in vitro formed shoots and their successful transplantation to the glasshouse with high frequencies.

Chickpea has been shown to be susceptible to wild *Agrobacterium tumefaciens* as well as *A. rhizogenes*, where infection results in the formation of crown galls and hairy roots, respectively, on the infected explant (Sonia et al., 2004). Even though the genetic transformation of chickpea has been reported where the primary transformants were confirmed by molecular analysis (Fontana et al., 1993; Kar et al., 1996, 1997; Krishnamurthy et al., 2000), the methods were genotype dependent and produced low frequencies of transgenic tissue or plantlets.

Krishnamurthy et al. (2000) used embryo axes devoid of root and shoot meristems for *A. tumefaciens*-mediated transformation of chickpea. The binary vectors contained two marker genes, the *uid*A gene expressing β-glucuronidase and the antibiotic selection marker *npt*II. After co-cultivation with *Agrobacterium* and selection on the antibiotic kanamycin, 16 shoots were obtained from nearly 4000 explants resulting in a transformation efficiency of 0.4%. Due to problems with efficient rooting, the shoots were grafted onto germinated seedlings of wild type chickpea prior to their hardening and transfer to soil. Genomic analysis was carried out for only 4 out of the 16 plants, which revealed the integration of single or multiple copies of T-DNA. The plantlets transferred to soil had reduced vigour and fertility, which the authors attributed to the suboptimal glasshouse conditions. Out of 36 plants growing in glasshouse, only 5 plants flowered and set seeds. T_1 progeny as analyzed by polymerase chain reaction (PCR) was found to be positive for the selection marker (*npt*II) but not for histochemical GUS assay measuring the β-glucuronidase activity.

A robust and reliable protocol for *A. tumefaciens*-mediated chickpea transformation has recently been demonstrated by Sarmah et al. (2004) using cotyledonary explants containing half embryogenic axes. The Australian 'desi-type' cultivar Semsen was transformed by using a bean gene construct that encodes for an α-amylase inhibitor and *npt*II as the selectable marker gene. Average transformation frequency was 0.72% and therefore higher than reported in previous studies (Krishnamurthy et al., 2000). However, since the transgenes were transmitted to the next generation by only 78% of the primary transgenics, transformation efficacy based on the functionally transformed explants was 0.56%. Southern blot analyses revealed that five out of six independent transgenic lines contained a single transgene insertion what is in contrast to earlier studies where multiple gene inserts were more frequently observed (Kar et al., 1996; Krishnamurthy et al., 2000).

At ICRISAT, chickpea transgenic lines have been developed by incorporating a synthetic *Bt cry1Ab* and soybean trypsin inhibitor gene through *A. tumefaciens*-mediated genetic transformation by using the regeneration protocol based on axillary meristem explants that produce adventitious shoots (Jayanand et al., 2003; K.K. Sharma, unpublished results). The molecularly characterised plants are currently in T_3 generation and being employed in insect bioassays for resistance to *H. armigera*. The protocol is applicable across a wide range of desi-type chickpea cultivars.

An overall view of the scenario of transformation in chickpea clearly demonstrates that considerable progress has been made in regeneration and genetic transformation of chickpea that had earlier remained a problematic crop for genetic engineering (Sonia et al., 2004). With this progress it is hoped that chickpea biotechnology will provide unique opportunities for its agronomic improvement against insect pests and other constraints to its productivity.

3.2. Candidate genes for genetic transformation of chickpea for insect resistance

3.2.1. Helicoverpa armigera

While a number of different types of insect-resistant genes are reported to encode for toxins that target

lepidopteran larvae and could thus be suitable to increase resistance of chickpea and other crops (Schuler et al., 1998; Hilder and Boulter, 1999), *Bt*-toxins still receive the most attention. *H. armigera* is sensitive to a range of *Bt*-toxins, with Cry1Ac being the most effective (Chakrabarti et al., 1998a; Kranthi et al., 2001; Liao et al., 2002). Synergistic activity of Cry1Ac and Cry1F toxins was also observed (Chakrabarti et al., 1998b). A recent study reported a five-fold variation in the sensitivity to Cry1Ac in *H. armigera* populations collected in different geographical regions in India (Jalali et al., 2004). So far, the only *Bt*-transgenic crop that has been commercialised for the control of *H. armigera* is cotton (James, 2002; Quaim and Zilberman, 2003). However, several other crops have successfully been transformed to express different *cry* genes for protection against this pest. These include tobacco (Selvapandiyan et al., 1998), brinjal (Kumar et al., 1998), potato (Chakrabarti et al., 2000) and tomato (Mandaokar et al., 2000; Kumar and Kumar, 2004). Two chickpea cultivars (ICCV 1 and ICCV 6) have been transformed to express the *cry1Ac* gene under a constitutive promotor (CaMV35S) through microprojectile bombardment using the *npt*II gene as the selection marker (Kar et al., 1997). Young shoots of T1 plants that expressed Cry1Ac at 0.004–0.0045% of soluble protein caused a feeding inhibitory effect on first-instar *H. armigera*. Unfortunately, the presence and expression of transgenes and resistance level in subsequent progenies have not been reported.

A number of studies have investigated the proteinase activity in the gut of *H. armigera* and the possible use of protease inhibitors (PIs) to control this pest. *H. armigera* has the highly alkaline midgut characteristics of Lepidoptera and secretes serine proteases as the main gut endoproteinases (Johnston et al., 1991). This explains the effects of serine PIs such as the soybean Kunitz trypsin inhibitor (SBTI) on *H. armigera* larvae in artificial diet bioassays (Johnston et al., 1991, 1993; Nandeesha and Prasad, 2001; Gatehouse et al., 2002) or when expressed by transgenic tobacco plants (Wu et al., 1997; Charity et al., 1999; Christeller et al., 2002; but see Nandi et al., 1999). Recent studies by Patankar et al. (2001) have also revealed the presence of metallo-, aspartic-, and cysteine-proteinase inhibitors in the larval gut. The authors reported a considerable diversity in the proteinase activity and a flexibility in their expression during the various developmental stages of the insect and depending upon the diet provided. The latter was suggested to be linked to the polyphagous nature of *H. armigera* which had evolved mechanisms to cope with different PIs that are encountered on the different host plants (Harsulkar et al., 1999). Further evidence for this is the fact that inhibitors of trypsin activity from host plants such as chickpea and pigeonpea (*Cajanus cajan*) do not have a strong effect on *H. armigera* (Godbole et al., 1994; Giri et al., 1998; Patankar et al., 1999). Such adaptations have also been reported for *H. armigera* feeding on artificial diets containing individual PIs (Bown et al., 1997; Gatehouse et al. 1997), or when feeding on transgenic tobacco plants expressing the giant taro proteinase inhibitor (Wu et al., 1997). Due to this adaptability, the use of PIs as a single resistance strategy must be considered carefully. One of the ways to use PIs may be to combine inhibitors that target different proteinases (Jongsma and Bolter 1997; Harsulkar et al., 1999), or preferably, to use PIs in alliance with other insecticidal gene products. For example, synergistic effects of soybean trypsin inhibitor and *Bt*-toxins on *H. armigera* have been reported by Zhang et al. (2000). The suggested mechanism was an extended retention time of the *Bt*-toxins in the insect's midgut.

Recently, transgenic tobacco plants expressing avidin, a protein that binds strongly to the vitamin biotin, have shown good protection against *H. armigera* (Burgess et al., 2002). Pyramiding the avidin gene with *cry1Ba* has resulted in a strong synergistic effect, while avidin expressing plants were as effective as plants that expressed avidin and a serine PI (aprotinin, BPTI).

At Assam Agricultural University, chickpeas have been transformed to express *cry1Ac* driven by either the constitutive CaMV35S promoter or a green tissue specific promoter (AraSSU). Presence and expression of the transgene are currently being confirmed by PCR and Western analyses (B.K. Sarmah, unpublished results). Research is currently in progress at ICRISAT to develop transgenic chickpeas expressing *Bt*-toxins, protease inhibitors, and lectin genes for resistance to *H. armigera* (Sharma and Ortiz, 2000; Sharma et al., 2002). Transgenic plants pyramiding *Bt*-genes (*cry1Ab* or *cry1Ac*) and the SBTI gene are at different stages of evaluation for resistance to *H. armigera* (K.K. Sharma and H.C. Sharma, unpublished results).

3.2.2. *Sap-sucking pests*

Until now, no transgenic plant expressing a resistance factor against sap-sucking pests has been commercialised. While there is a patent available on *Bt*-toxins that affect aphids (Payne and Cannon, 1993), there is no published evidence for this. The focus of research has therefore been on other insecticidal compounds. Plant-derived lectins appear to be the most promising candidates. Lectins are carbohydrate-binding proteins that are thought to play a defensive role in plants in response to attack by herbivores or pathogens (Peumans and van Damme 1995). So far, transgenic crops expressing lectins from snowdrop (*Galanthus nivalis*, GNA) (Shi et al., 1994; Down et al., 1996; Stoger et al., 1999; Foissac et al., 2000), jackbean (*Canavalia ensiformis*, ConA) (Gatehouse et al., 1996) or wheat (*Triticum vulgaris*, WGA) (Kanrar et al., 2002) have shown partial resistance to aphids (Aphididae), leafhoppers

(Cicadellidae) and planthoppers (Delphacidae). Besides the lectins that have been expressed in transgenic plants, a number of other lectins have been found to affect sap-sucking insects when provided in artificial diets (Habibi et al., 1993; Rahbé et al., 1995; Sauvion et al., 1996; Bandyopadhyay et al., 2001; Roy et al., 2002). Because many of the sap-suckers are phloem-feeders, studies are in progress to target the transgene products to the phloem-sap by the use of phloem-specific promoters such as the rice sucrose synthase promotor (RSs1) (Shi et al., 1994).

In aphids, lectins appear to act primarily by reducing growth, development, and fecundity rather than causing mortality. This makes the additional impact of antagonists such as predators and parasitoids necessary for pest control (Van Emden, 1999). In addition, some studies have suggested a 'feeding deterrent' effect of lectins (Shi et al., 1994; Powell et al., 1995; Kanrar et al., 2002), which appears to be a consequence of intoxification rather than sensory-mediated (Sauvion et al., 2004). Changes in the insect feeding behaviour could potentially increase virus spread due to an increase in the frequency of plant visits and probing by the vectors, which is especially likely for non-persistant viruses (Kennedy, 1976). Since both the bean leaf roll luteovirus and the chickpea chlorotic dwarf virus are transmitted by their vector in a persistent manner (Horn et al., 1994; Brunt et al., 1996), a feeding deterrent effect of lectin-expressing transgenic chickpeas might not cause an increase in virus spread (Kennedy, 1976). However, this would need further investigation once lectin-expressing chickpea plants are available since virus spread will depend on the amount of time an aphid spends feeding on the plant and the inoculation and retention times of the virus. For example, the minimum acquisition access period reported for *O. orientalis* was found to be only 2 mins (Horn et al., 1994). Also, chickpea stunt is caused by a number of different viruses (Horn, 1994), for which the mode of transmission is not yet known. If chickpea diseases cannot be controlled by enhancing resistance towards the vector, plants could be genetically engineered to target the virus directly (Schillberg et al., 2001; Dasgupta et al., 2003). However, this approach might not be durable since viruses evolve rapidly (Prins, 2003).

So far, only a few mannose-binding lectins have been tested for their activity against *A. craccivora* in artificial diet bioassays: leaf lectins from garlic (*Allium sativum*, ASAL), onion (*Allium cepa*) and *Diffenbachia sequina* as well as a lectin from tubers of *Colocasia esculenta*. Of the four lectins tested, ASAL was found to be the most effective against *A. craccivora* [with a median effective dose (LC_{50}) of 0.15 nmol], followed by that of onion, *C. esculenta* and *D. sequina* (Sampa Das, unpublished results). After incubating *A. craccivora* on an artificial diet containing a sublethal dose of ASAL, the aphid's midgut was dissected and challenged with anti-ASAL antibodies. Light microscopic observations demonstrated the binding of the lectin to the inner epithelial membrane of *A. craccivora* which may explain the insecticidal activity of ASAL. At least some of the mannose binding lectin ASAL appears to remains stable when passing through the gastrointestinal tract, at least to the extent that immunoreactive peptides are detectable. This is important since biochemical stability is a prerequisite for biological activity of the lectin. ASAL has earlier been shown to be effective against sap-sucking pests, i.e. the aphid *Lipaphis erysimi* Kaltenbach (Hemiptera: Aphididae) and the red cotton bug *Dysdercus cingulatus* (Fabricius) (Hemiptera: Pyrrhocoridae) (Bandyopadhyay et al., 2001, Roy et al., 2002). ASAL is therefore a potential agent to control *A. craccivora* and chickpea transformation work has commenced using the lectin coding sequence driven by either a constitutive (CaMV35S) or phloem-specific (RSs1) promoter (Sampa Das, unpublished results).

3.2.3. Bruchids

While a number of plant derived lectins have been shown to affect *Callosobruchus* species when provided in artificial seeds, those with specificity for *N*-acetylgalactosamine/galactose or *N*-acetylglucosamine seem to be the most effective in inhibiting larval development (Murdock et al., 1990; Gatehouse et al., 1991; Zhu et al., 1996; Huesing et al., 1991a,b; Machuka et al., 2000; Macedo et al., 2002b).

Bruchids use cysteine proteinases as their predominant digestive enzymes with an optimum activity at about pH 5 (Ryan, 1990), explaining the reported effects of protease inhibitors (PIs) of the cysteine group (Murdock et al., 1988; Campos et al., 1989; Kuroda et al., 1996; Oliveira et al., 2001a, 2002; Macedo et al., 2002a). In addition, activity of aspartic proteinases has been reported in *C. maculatus* larvae (Silva and Xavier-Filho, 1991). The reported effects of the cowpea trypsin inhibitor on growth and development of *C. maculatus* larvae (Gatehouse and Boulter, 1983) is due to the fact that this PI shows a low level inhibitory activity against cysteine type proteinases (Gatehouse et al., 1985). Recently, Zhu-Salzman et al. (2003) reported a synergistic delay in development of *C. maculatus* by a recombinant fusion protein consisting of the soybean cysteine protease inhibitor soyacystatin and *Griffonia simplicifolia* lectin II, whereas a mixture of the separate proteins only showed an additive effect.

A range of other compounds isolated from legume seeds have shown insecticidal activity to *Callosobruchus* spp. These include polysaccharides (Applebaum et al., 1970; Oliveira et al., 2001b), proteins with unknown functions such as canatoxin (Carlini et al., 1997) and zeatoxin (Macedo et al., 2000), and storage proteins such as vicilins (Macedo et al., 1993; Yunes et al., 1998).

By far the most effective compounds are those lectin-like proteins that inhibit the insects α-amylases, enzymes that catalyse the hydrolysis of α-1,4-glucan linkages in starch components, glycogen and other carbohydrates. Such inhibitors (α-AIs) with activity against *Callosobruchus* spp. have been purified from different plant sources (Franco et al., 2002). The best characterised inhibitor, α-AI-1, has been purified from the common bean *Phaseolus vulgaris* and the cDNA has been cloned (Moreno and Chrispeels, 1989; Chrispeels et al., 1998). α-AI-1 is known to inhibit the amylases of three important Old World bruchid pests. Bioassays deploying artificial seeds revealed that the presence of α-AI-1 at a level of 0.2% (w/w) almost completely inhibits the development of larvae of *C. chinensis* and *C. maculatus* (Ishimoto and Kitamura, 1989). In addition, transgenic peas expressing α-AI-1 have shown good protection against *Bruchus pisorum* (L.) (Schroeder et al., 1995). For comparison, the α-amylases of the Mexican bean weevil *Zabrotes subfasciatus* (Boheman) are not inhibited by α-AI-1 (Ishimoto and Kitamura, 1989). While α-AI-1 is inactive against plant and bacterial enzymes, it also inhibits mammalian α-amylases (Chrispeels et al., 1998). Therefore this anti-nutritional factor must be inactivated by cooking prior to human consumption. Inhibitors that affect *Callosobruchus* spp. α-amylases but not those of mammals have also been reported (Yamada et al., 2001; Franco et al., 2002). For example, some wild accessions of the common bean contain an inhibitor (α-AI-2) that exclusively inhibits insect α-amylases. While α-AI-2 inhibits the α-amylases of *Z. subfasciatus*, it is not effective against *C. maculatus*, and far less active against *C. chinensis* when compared to α-AI-1 (Suzuki et al., 1993).

Peas (*Pisum sativum* L.) (Shade et al., 1994) and adzuki beans (*Vigna angularis*) (Ishimoto et al., 1996) were genetically modified to express the bean cDNA encoding α-AI-1. A α-AI-1 gene construct was used that is regulated by flanking sequences from the seed-specific bean phytohemagglutinin (PHA-L) gene (*dlec2*). The PHA-L regulatory DNA sequences restrict the expression of the α-AI-1 to the cotyledon and embryonic axis of the developing seeds (Schroeder et al., 1995). Transgenic peas were tested for their resistance to *C. maculatus and C. chinensis* in the laboratory (Shade et al., 1994). For *C. chinensis*, in nearly every seed where α-AI-1 was detected, all infesting larvae died. Seeds from which adults emerged contained a maximum of 0.14% (w/w) of the inhibitor. The response of *C. maculatus* was more variable and depended on the level of α-AI-1 in the seeds, with levels of above 0.8% giving complete mortality. Subsequent experiments showed that α-AI cDNA was expressed stably in transgenic pea seeds at least to the T_5 generation (Schroeder et al., 1995). Transgenic peas in which the α-AI-1 level reached approximately 3.5% of the total protein, provided complete protection against *B. pisorum*, both in greenhouse as well as in field studies (the pea weevil lays its eggs on developing green pods) (Schroeder et al., 1995; Morton et al., 2000). Larval development was found to be blocked at an early developmental stage (Schroeder et al., 1995). Studies with transformed adzuki beans that expressed α-AI-1 in their seeds at a level of 0.9% dry weight were found to be completely resistant to three species of *Callosobruchus* spp. but not, as expected, to *Z. subfasciatus* (Ishimoto et al., 1996). Peas expressing α-AI-2 in their seeds were not protected from *B. pisorum* (Morton et al., 2000).

Transgenic chickpea lines that express α-AI-1 in their seeds were developed using an Australian cultivar. The α-AI-1 produced in chickpeas was shown to be functionally active against α-amylase from porcine pancreas in vitro. Transgenic chickpeas expressing α-AI-1 at a level of 2.1% of seed protein inhibited the development of *C. maculatus* and *C. chinensis* by over 90% in insect bioassays (Sarmah et al., 2004). Thus the α-AI-1 gene is considered as effective for developing transgenic chickpeas resistant to storage pests. Presently, a transformation system has been established for Indian chickpea cultivars (Das et al., 2002) and transgenics are being developed using a reconstructed bean α-AI-1 gene (B.K. Sarmah, unpublished results).

4. Possible effects of insect-resistant GM chickpeas on entomophagous arthropods

Among the widely discussed environmental impacts of GM crops is their potential effect on non-target organisms including entomophagous insects (Dale et al., 2002; Conner et al., 2003). Since parasitoids and predators are important for natural pest regulation, they help to prevent secondary pest outbreaks and can affect the rate of resistance evolution of the target pest to the introduced resistance factor (Gould et al., 1991; Gould, 1994). A risk assessment should therefore be carried out for the economically or ecologically most important species associated with the crop prior to the commercial release of the novel plant. Such a regulatory testing and risk assessment is well established for pesticides (Candolfi et al., 2001) and there are many lessons to learn for risk assessment of GM plants (Hill and Sendashonga, 2003). Recently, Dutton et al. (2003) have proposed a tiered testing procedure for insect-resistant GM plants with increasing levels of complexity and realism that has largely been adapted from the ecotoxicological evaluation of pesticides. As a first step, it is required to determine which entomophagous arthropods play an important role in regulating pests in the respective GM crop. Since risk is defined as a product of a hazard (toxicity of a transgene product) and exposure (level of exposure to the compound), it has

to be established which of the selected arthropods are potentially exposed to the transgene product under field conditions in a second step. For the arthropods that are likely to be exposed, their sensitivity to the product then has to be established (hazard identification and characterisation). The potential hazard of the insecticidal proteins will vary with their spectrum of activity. While *Bt*-toxins are known to be active on a subset of species belonging to the same insect order, other compounds such as lectins or protease inhibitors have a less-specific mode of action, potentially affecting many more non-target species (Schuler et al., 1998; Hilder and Boulter, 1999). Based on the information gained, the potential risk of a certain GM plant on non-target organisms can be characterised and possibly managed (Hickson et al., 2000; Dutton et al., 2003).

The application of this approach to crop systems in tropical areas, where the arthropod communities are usually not well described, is a challenge. In the following, we discuss possible routes through which entomophagous insects could be exposed to insecticidal proteins expressed by GM chickpeas. We further recommend species that could be selected for a pre-release risk assessment taking the availability/amenability and the knowledge on the species' biology into account since these are important prerequisites for setting up reliable and reproducible bioassays.

Even though a number of insect herbivores have been reported to be associated with chickpea (Van Emden et al., 1988; CPC, 2001), we will confine our focus on the three major pests in India, i.e. *H. armigera*, aphids and bruchids (Fig. 1). This is done, firstly, because these are the only chickpea herbivores for which information regarding their antagonists is available, and, secondly, any disturbance of the natural control of the herbivores that are not targeted by a particular GM chickpea should be avoided. There are a number of routes through which entomophagous arthropods could be exposed to the transgene products. (i) *Carnivory of herbivorous arthropods*—The predominant route for entomophagous insects to be exposed to transgene products is through their prey or hosts. Therefore it has to be assessed at an early stage which herbivores ingest the transgene product when feeding on a GM chickpea. This will depend on the mode of feeding of the herbivores and on the site and time of toxin expression (Dutton et al., 2003). The latter will vary with the promoter that drives the transgene. *Bt*-chickpeas that are currently developed express the Cry1Ac toxin under a constitutive (CaMV35S) or green tissue specific (Ara SSU) promoter, expression of α-AI-1 is restricted to the seeds while it is envisaged to express ASAL under a phloem-specific (RSs1) promoter. In addition, concentration of a transgene product within an insect can also vary among species with a similar mode of feeding (Foissac et al., 2000; Head et al., 2001). (ii) *Direct plant feeding*—A number of predators are facultative feeders on plant material (incl. pollen) (Coll and Guershon, 2002). While transgene products have so far not been detected in nectar of GM crops (Malone and Pham-Delègue, 2001), pollen may contain the novel proteins when constitutive promoters such as CaMV35S are used (for *Bt*-maize see Dutton et al., 2003). (iii) *Honeydew feeding*—Transgene products have been detected in the sugar-rich excretions (honeydew) from phloem-sucking Sterrnorhyncha (Hemiptera) feeding on insect-resistant transgenic plants. This indicates that the transgene product was present in the phloem-sap and could potentially affect a broad range of non-target arthropods including entomophagous insects that use honeydew as an energy source (Romeis et al., 2003). The presence of transgene products in honeydew has been observed not only in cases where the trangenes were driven by a phloem-specific promoter such as RSs1 (Shi et al., 1994) but also in cases where a constitutive promoter was deployed (Kanrar et al., 2002; Bernal et al., 2002; Rahbé et al., 2003).

In addition, transgene products also enter the soil ecosystem either through crop residue or herbivore detritus and can be detectable in the soil for a long period of time resulting in exposure of non-target soil organisms to the compound. The effects of GM plants on soil communities and processes in soil have been reviewed recently by Bruinsma et al. (2003) and Kowalchuk et al. (2003).

The entomophagous arthropods that attack *H. armigera* in India are relatively well known (Fig. 1). However, information regarding their impact and activity in the chickpea crop is still scarce (Romeis and Shanower, 1996). From the parasitoids reported to attack *H. armigera* on chickpea, *Campoletis chlorideae* is particularly abundant and effective (Romeis and Shanower, 1996). This along with the fact that *C. chlorideae* can be reared in the laboratory and is relatively well studied (e.g. Nikam and Gaikwad, 1991; Murugan et al., 2000) makes it a possible candidate for risk assessment studies. Only a few predatory arthropods are reported from chickpea (Mehto et al., 1986; Singh et al., 1990) (Fig. 1). One of the species, the ladybird beetle *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), is a well established test insect for ecotoxicological studies (Schmuck et al., 2000). This species is known to attack both lepidopteran larvae as well as aphids (including *A. craccivora* on chickpea; Saxena et al., 1970), making it a good test species. Several other aphid predators have been reported in India, although not from chickpea (Waterhouse, 1998; Joshi et al., 1997). There is no published information on the parasitoids that attack aphids on chickpea, and field investigations should be conducted to describe the parasitoid complex involved. In the meantime, one might have to consider species that attack the chickpea infesting aphids on

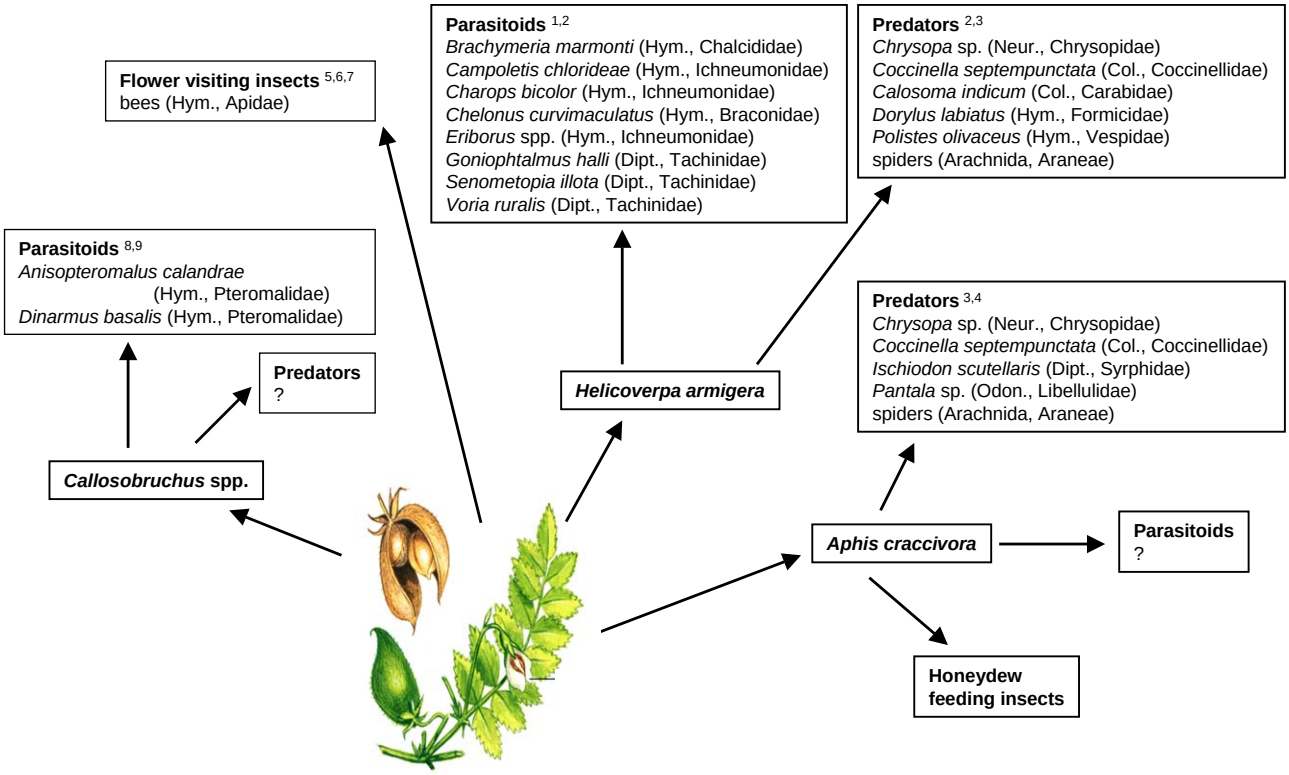

Fig. 1. Trophic interactions in the chickpea crop in India (1—Romeis and Shanower, 1996; 2—Singh et al., 1990; 3—Mehto et al., 1986; 4—Saxena et al., 1970; 5—Ayyar, 1935; 6—Niknejad and Khosh-Khui, 1972; 7—Reed et al., 1987; 8—Devi, 1996; 9—Verma, 1991).

other crops for risk assessment studies. Parasitoids reported to attack *A. craccivora* in India have been listed by Waterhouse (1998). Indigenous parasitoids that might be suitable test species are the well studied *Trioxys indicus* Subba Rao and Sharma (Hymenoptera: Aphidiidae) (Singh and Agarwala, 1992) and *Aphidius colemani* Viereck (Hymenoptera: Braconidae) (Starỳ, 1975). In general, little is known about the arthropods attacking bruchids in storage (Van Huis, 1991). The only parasitoid that has been reported from chickpea samples collected in storage systems in India is *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Devi, 1996). Another parasitoid species that could be deployed in risk assessment studies is *Dinarmus basalis* (Rondani) (Hymenoptera: Pteromalidae). This species is reported to occur in India and has successfully been reared on chickpea seeds infested with different *Callosobruchus* spp. (Verma, 1991). Furthemore, both parasitoid species can easily be reared and are well described.

5. Conclusions

Insect-resistant GM chickpeas have the potential to reduce current gaps between attainable and actual yields, especially in subsistence farming systems, and to decrease the dependency on pesticides in some circumstances. Benefits will be maximum in the control of *H. armigera* since this pest is difficult to control with conventional methods usually requiring numerous pesticide applications. However, it appears that no single approach will suffice in itself to control *H. armigera*. Pyramiding different insecticidal genes (e.g. Zhang et al., 2000; Burgess et al., 2002) and the integration of transgenics with other components of pest management have to be envisaged (Waage, 1996; Fitt, 2000). The expression of the α-amylase inhibitor α-AI-1 in chickpea seeds has the potential to give good protection against bruchid attack. But this single resistant gene will need to be supplemented by other methods of pest control since it poses a high selection pressure that is likely to cause the rapid emergence of bruchid strains that are not affected by the inhibitor. It remains to be seen whether aphid-transmitted viral diseases can be controlled by regulating the aphid vector, for example by the deployment of plants that express lectins such as ASAL. The success of this approach will largely depend on the effectiveness of the transgene product and on the nature of the virus such as virulence and the speed at which it is transmitted.

One major obstacle to the deployment of transgenic chickpeas expressing *cry* genes from *Bacillus thuringiensis* for the control of *H. armigera* might be the

development of resistance in the target pest. Susceptible larvae of *H. armigera* are already not very sensitive to *Bt*-toxins such as Cry1Ac (Akhurst et al., 2003) when compared to other Lepidoptera and strains with high levels of resistance to this particular *Bt*-toxin have already been selected (Kranthi et al., 2000; Akhurst et al., 2003). But despite the fact that the deployment of *cry1Ac* expressing *Bt*-cotton in China increased from 10,000 ha in 1997 to 1 million ha in 2000, no increase in the resistance of *H. armigera* to Cry1Ac was detected (Wu et al., 2002). Implementing a resistance management plan in a country such as India may be difficult. Since *H. armigera* is a polyphagous pest with a reported number of at least 181 host plants from 45 families in India alone (Manjunath et al., 1989), other hosts that do not produce *Bt*-toxins could act as refuges and continuously produce susceptible insects. However, this effect will be lessened when the same Cry toxins (or those with cross-resistance) are expressed in different food plants of this pest. This is for example the case for *cry1Ac* and *cry1Ab* that are expressed in the commercialised *Bt*-cotton variety and currently engineered not only in chickpea, but in a number of other crops including pulses, vegetables and cereals (Sharma et al., 2003a). The deployment of these crops would increase the selection pressure on *H. armigera* requiring a more adaptive strategy including effective and sensitive resistance monitoring (Andow, 2002). A preventive measure to delay the development of resistance could be the pyramiding of dissimilar *Bt*-toxin genes (Zhao et al., 2003).

Another concern related to the deployment of GM crops is the possible gene flow to non-transformed varieties and wild relatives. However, this risk is low since chickpea is a strong self-pollinator. Pollination occurs when the keel is still closed, 12–24 h before the flower is fully expanded (Ayyar, 1935; Eshel, 1968). In addition, viability of the pollen is decreased when the flowers are fully expanded (Eshel, 1968). Field studies have established that natural out-crossing to other varieties occurs at levels below 1%, probably due to pollen being transported by flower visiting insects (Van Rheenen et al., 1990; Tayyar et al., 1995). Based on this knowledge, an isolation distance of 5 m for certified seed crops in India has been established (Chowdhury et al., 1991).

Besides concerns relating to environmental and health risks, issues relating to the intellectual property rights and corporate dominance have led to limited acceptance of GM crops among the public and policy-makers in developing countries (Paarlberg, 2001). This could partly be overcome when GM crops are developed in public research institutions. Currently, a number of active plant transformation programmes are in progress in public research institutions in India including ICRISAT. The latter can also play an important role in facilitating technology transfer by fostering North–South partnerships in developing countries (Morris and Hoisington, 2000; Sharma et al., 2003a).

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