DISCUSSION

Genetic potential of a crop mainly determines its quality and productivity. Other major factors that affect the quality and productivity of a crop are environmental stresses. There are varied ways in which environmental stresses can exert their influence on plants yet the most common form of stresses that affect plants is availability of nutrients. Exploring the different inherent mechanisms underlying plant tolerance to environmental stresses is therefore essential for formulating strategies for crop improvement under different stress conditions. Loss of nutrients from agricultural fields leading to poor nutrient availability often limits crop yield and quality. Balanced fertilization often leads to overall crop improvement in terms of yield, growth and quality. Nutrient form and timing more often than not also determines the nutrient uptake by crops. The response of some medicinal plants to plant growth regulators have also been observed to be significant in achieving desirable characters in the crops. Exogenous application of small amounts of plant growth regulators can also affect crop quality and yield.

Practitioners of traditional medicine of yester years collected certain herbs based on their specific characteristics mainly morphological attributes flowers, fruits, leaves etc. Drugs of specific color, size, smell, fibrous content, itching quality etc. were preferred over others and were collected from untrodden locations unknown to people. Even within a single medicinal plant species, some varieties were favored over several others. It was also a common knowledge to these practitioners that season and developmental stage alter the curative properties of these herbs and hence required plant or their parts were harvested during certain preferred periods only (Bharat, 1997). The variations in phenotypes prevalent in different populations of a single species of plant were perceived to result from environmental variations by these early physicians and botanists (Briggs and Walters, 1984). This problem (variations in phenotypes) in medicinal plants is intensified further because these plants not only show perceptible phenotypic variations but also synthesize and amass a diverse range of plant-specific chemicals. It is these biosynthetic derivatives of primary metabolism (Secondary metabolites or phytochemicals), accumulated by plants in certain specific parts in trace amounts, that form the entities of interest from these medicinal plants. These secondary metabolites in plants form an alterative armory to ward off biotic and abiotic stress induced effects on the plant body. Therefore studying the intra-specific variations in the active
principles in different seasons and at different plant growth stages forms a significant element in studies leading to deciphering the variations in these plants.

Despite the tremendous economic and medicinal significance of *A. paniculata* no efforts have gone into its cultivation in any part of the country. The plant is wholly or largely harvested by practitioners and drug companies from wild sources, which has put a lot of pressure on the natural populations of this plant. Selection of high-yielding accession(s) from the existing germplasm therefore forms an ideal option for making cultivation of *A. paniculata* environmentally favorable and economically viable, and also for determining the effect of addition of nutrients like nitrogen and sulfur and plant growth regulators like 24-epibrassinolide and ethylene for its cultivation on a larger scale.

The objective of the first experiment was to screen out one high yielding and one low yielding accession of *A. paniculata* in order to observe how the identified accessions responded to the applied nutrients and plant growth regulators.

5.1. **Screening of different accessions for yield and yield related parameters**

5.1.1 **Herb fresh weight and Shoot height**

The primary focus of the first experiment was to screen 14 different accessions of *A. paniculata* on the basis of their NR activity, overall yields and the yield of major active principle andrographolide. Significant variation was observed in the different accessions in terms of fresh weight and plant height. This is in conjunction with the studies of Anjana et al. (2007) on wheat, Naidu et al. (2000) on garlic and Bhan et al. (2006) on *A. paniculata*. Variable fresh weight and shoot height result from genotypic differences and their interactions with environment and other growth conditions including nutrients (Shivay et al., 2003; Woodend et al., 1987). Plant height kept on increasing with the plant age as expected in all the accessions primarily due to cell division and elongation which continues throughout the growing period of a plant. Fresh weight increased up to the flowering stage in all the accessions and peaked at just after flowering after which it decreased due to leaf fall in the plant. This is consistent with several earlier studies on the same plant (Parashar et al., 2011; Pholphana et al., 2013).
5.1.2 Total lactone and andrographolide content

The accessions also showed a significant variation in terms of total lactone and andrographolide content which was again consistent with the studies of Bhan et al. (2006) on the same plant. Intraspecific variation in the yield of active principles has been noted in several other plants as well (Broekgaarden et al., 2010; Fincher et al., 2008; Kelman et al., 2000; Luengas-Caicedo et al., 2007; Puglisi and Paul, 1997). The variation in the quality and quantity of plant chemicals are explained by plant ontogeny and genetic and environmental variations (Moore et al., 2014). It has been observed that plant secondary metabolites vary at species scale, population scale, and individual scale and even at the scale of plant parts (Holeski et al., 2012; Suomela et al., 1995). Variations in the active principles of plants can be attributed to the genetic makeup, environment and their interaction which extends to plant ontogeny and phenology. The concentrations of several plant secondary metabolites vary significantly across environments (physical as well as biotic) due to environmental adaptability or genotypic sorting and selection within different habitats. Most of this variation arises due to differences in phenotypic malleability of different genotypes to variations in resource (light, water and nutrients) availability for growth and the absence or presence of competitors and enemies. The resultant changes in the amount of plant secondary metabolites might occur due to down- or up-regulation of their biosynthesis (Koricheva, 1999). Plant tissues are defended according to their value to the plant, the level of threat of herbivory and the costs of defense (Rhoades and Cates, 1976). All these quadrants change throughout the life span of plants, and therefore the plant secondary metabolite concentrations (Barton and Koricheva, 2010). Both andrographolide and total lactone contents increased with plant age and reached a plateau at the flowering stage after which their contents started to decline. This was consistent with earlier studies on the plant (Parashar et al., 2011; Pholphana et al., 2013). The decrease in the contents of these active principles could have possibly been due to a decrease in their needs and their subsequent catabolism once the leaves started shedding, or their partial translocation into the grain at the post-flowering stage.

5.1.3 Nitrate reductase activity

Nitrate reductase activity has been used as a biochemical marker for the identification of superior (higher herbage yielding) germplasm in several plants (Eilrich and Hageman, 1973; Sarma et al., 2011). The accessions under study displayed a significant variation in terms of
nitrate reductase activities. NR activities were higher in high yielding accessions and were lower in low yielding accessions displaying a significant positive co-relation. Similar results demonstrating natural variation in nitrogen metabolism, and nitrate uptake and remobilization in the plant have also been obtained in several other plants like lemon grass (Sarma et al., 2011), wheat (Anjana et al., 2011), maize (Reggiani et al., 1999), and agrostis (Osborne and Whittington, 1981). NR activities were optimum at the pre-flowering stage and decreased gradually with the age of the plant. This is again consistent with the findings of Pandey et al. (2012) in Avena species, van Berkum and Sloger (1981) in rice and Anjana et al. (2006) in several leafy vegetables. Observed differences in NR activities in the different accessions of the plant were inherent (genetic) in the plants (Reggiani et al., 1999).

After screening out two accessions (HYA and LYA) our next aim was to study the response of identified accessions to different levels of applied nutrients (N and S) and plant growth regulators (24-epibrassinolide and ethylene). It was observed that the two accessions responded differently to the applied nutrients and plant growth regulators. This might have happened due to the inherent differences in their nutrient uptake and utilization efficiencies and their general responsiveness to applied plant growth regulators which have been observed to be highly variable among different plant species.

While research focused on the effect of nutrient (N and S) application and the application of plant growth regulators (24-epibrassinolide and ethylene) on crop plants is present in abundance, these aspects remain mostly and to a high extent, unexplored in the medicinally important A. paniculata. Nitrate as a nutrient, is taken up through the root systems from the soil with the aid of nitrate transporters. The nitrate taken up by the roots is the subsequently converted to ammonium by the successive action of NR and NiR. The resultant ammonia is then used to synthesize amino acids by the sequential action of GS, GOGAT and GDH. NUE, which at plant level is the ability of a plant to utilize available N resources to maximize its productivity, is critical not only for crop quality and quantity but also for maximal nutrient utilization. While N availability to plants can be increased by different nutrient and crop management strategies as discussed in the preceding sections, the internal N utilization efficiency needs to be tackled biologically (Abdin et al., 2005). NUE, as already discussed, is a complex quantitative trait and depends on several external and internal factors, such as the availability of other nutrients like sulfur and plant growth regulators (Anjana et al., 2007).
Nitrogen is the most important element to plants with regard to its concentration, biochemistry and physiological functions, second only to water (Russel and Williams, 1977). N is an essential constituent of amino acids and hence regulates protein synthesis. N is essential for plant growth and reproduction. It is a component of chlorophyll and is therefore necessary for photosynthesis. N is a basic constituent of animal and plant proteins, including the genetic material DNA and RNA, and is essential at times of rapid plant growth (van Oijen and Levy, 2004).

Sulfur is an important element for plant growth and its requirements in the plant are closely related to the nitrogen availability and plant growth (Coleman, 1966; Hawkesford, 2007). S performs numerous vital functions within plants. In-organic S is transformed into vital (nutritionally and functionally) S-containing amino acids such as cysteine and methionine which are important constituents of proteins, cofactors and secondary products, through a network of enzyme controlled reactions (Koprivova et al., 2008). The formation of sulphydryl and disulfide bonds which work in protein stabilization. Thiol groups also form the active centers in several enzymes (Saito, 2000). Thiol groups as parts of cysteine molecule form strong nucleophiles making them perfectly suitable for different redox reactions in biological systems (Nazar et al., 2011).

Brassinosteroids (now treated as plant hormones) possess a significant growth-promoting activity through increased cell elongation and cell division (Cutler et al., 1991; Yopp et al., 1981). Brassinosteroids have also been shown to influence several other developmental processes like flowering and reproduction (Pipattanawong et al., 1996; Rao et al., 2002), vascular development (Adam and Petzold, 1994), membrane polarization and proton pumping (Chory et al., 1996; Davies, 1995), seed germination (Evenari, 1949; Jones-Held et al., 1996), senescence, abscission and maturation (Mandava et al., 1981). Brassinosteroids have also been proven to confer resistance and to ameliorate the adverse effects of various abiotic stresses in plants. Brassinolides are also known to interact with environmental signals thereby affecting insect and fungal development (Bajguz and Hayat, 2009; Clouse and Sasse, 1998). Different processes of plant growth and photosynthesis are influenced by ethylene as well (Khan and Khan, 2014; Khan, 2004a). Ethylene also plays an important role in the resistance of plants towards abiotic stress (Khan et al., 2012; Khan and Khan, 2014). Ethylene has a role in sulfur metabolism (Asgher et al., 2014), proline metabolism (Khan et al., 2013), nitrogen metabolism (Khan et al., 2008), antioxidant metabolism (Khan et al.,
2014), and osmolyte function (Khan et al., 2012; Khan et al., 2014; Khan et al., 2013), under both normal and stressful environments. Ethylene is has been shown to have an influence on the photosynthetic processes through altered stomatal conductance (Khan et al., 2014), or through the allotment of S and N Rubisco protein (Asgher et al., 2014; Khan and Khan, 2014), or through the regulated production of osmolytes (Khan et al., 2012; Khan et al., 2014; Khan et al., 2013). In addition to this ethylene also influences fruit firmness and color development (Dhillon and Mahajan, 2011). Here forth we will discuss the effect of application of these nutrients (N and S) and plant growth regulators (24-epibrassinolide and ethylene) on the growth, biochemical characteristics, yield, N metabolism and NUE in the two selected (HYA and LYA) accessions of *A. paniculata*.

### 5.2 Growth characteristics

#### 5.2.1 Shoot length, number of nodes per plant and 10th internode length

Increasing concentrations of N, S and 24-epibrassinolide led to significant increase in the shoot length of the plants. Among the two accessions V3 showed a tendency towards more vigorous growth under all treatments and treatment ratios. Similar results were depicted when factors like treatments and accessions were interacted. These results were in accordance with earlier studies which observed increased plant height with the application of N (Ayub et al., 2011; Delfine et al., 2005; Verma et al., 2010; Wang et al., 2008), S (Ali et al., 2012; Jamal et al., 2005; Jeet et al., 2012) and 24-epibrassinolide (Arora et al., 2010a; Sharma and Bhardwaj, 2007; Swamy and Rao, 2009) in different plants. Application of ethylene did not result in any significant increments in plant height, number of nodes or the length of the 10th internode in any of the two accessions of Kalmegh under study. It rather led to a slight decrease in the shoot length in the two accessions. These results are in agreement with the findings of Burg (1973) and Reid (1995) which reported significant decreases in plant height with increasing ethylene concentrations.

The increase in shoot length with the application of N, S and 24-epibrassinolide could be primarily attributed to cell elongation and cell division resulting in increase in the lengths of internodes and the number of nodes and internodes in both the accessions of the plant. Hussain et al. (2006) found that nitrogen addition significantly increased plant height in *Alpinia galanga*. Addition of S has also been shown to increase plant height in wheat (Ali et al., 2012; Jeet et al., 2012) as does an interaction between the two (Jeet et al., 2012). Addition
of growth stimulator 24-epibrassinolide has also been shown to positively affect plant height under both normal and stress conditions (Houimli et al., 2008; Liu et al., 2014; Shahid et al., 2011). Shoot length, number of nodes per plant and the length of 10th internode kept on increasing with the age of the plant in controls as well as at different doses of applied nutrients and plant growth regulators as expected. The results are again consistent with the studies of Parashar et al. (2011) on A. paniculata which noted significant increase in the these parameters with the age of the plant.

5.2.2 Fresh weight

Nitrogen application has been reported to improve plant growth in a majority of plants (del Amor and Cuadra-Crespo, 2011; Kaya and Higgs, 2003; Masaka and Chivandi, 2005; Orloff et al., 2012). In the present study, it was observed that nitrogen application significantly increased plant growth indicated by the increase in plant fresh weight as in previous reports (Chen et al., 2010; Dong et al., 2010; Jabeen and Ahmad, 2009). Increased rates of sulfur also significantly increased the biomass in the both the accessions of the plant in our study. The results obtained were in agreement with earlier studies on different crop plants (Isuwan et al., 2007; Kulczycki, 2011; Zhong et al., 2011). The increase in biomass with the application of N and S can be attributed to cell expansion, elongation and division leading to taller plants with increased number of leaves and branches and consequently higher fresh weights. S availability is also known to influence NUE in plants and vice versa (Fismes et al., 2000; Schnug, 1991; Schnug et al., 1993). Availability of sulfur is known to interact with N availability there by affecting the management of the two nutrients and consequently the biological yields (Janzen and Bettany, 1984; Kopriva and Rennenberg, 2004). Application of plant growth regulators 24-epibrassinolide and ethylene also led to significant increase in the plant biomass of both the accessions up to varying degrees. Similar results of epibrassinolide application were also obtained by Naeem et al. (2012) in Mentha arvensis, Hayat et al. (2011) in tomato and Swamy and Rao (2009) in Pelargonium graveolens. Exaggerated growth in Arabidopsis (Arteca and Arteca, 2001) and Chlorella vulgaris (Bajguz and Czerpak, 1998) by epibrassinolide application is also on record. 24-epibrassinolide increased biomass in the plants through increased cell division and elongation (Arora et al., 2010b). Brassinolides are potent growth stimulants in vegetative tissues (Arora et al., 2010b). They have been reported to increase the growth of mung bean, pea epicotyls, soy bean, sunflower, bean, Arabidopsis peduncles, cucumber hypocotyls and Hordeum vulgare seedlings (Clouse, 2008; Clouse et
Brassinolides have also been shown to up-regulate the expression of auxin regulated GH1 and SAUR genes, however, the kinetics of regulation of the two genes differed between auxins and brassinolides (Clouse and Sasse, 1998). It is noteworthy to mention here that GA’s and auxins promote cell elongation in plants. The increase in fresh weights in the two accessions of the plant in our study by ethylene application could have been due to cell expansion and elongation rather than division. Such results have also been obtained in earlier studies of Khan et al. (2008), Khan (2003), Khan (2004a) and Khan and Khan (2014) on different varieties of mustard. In all the treatments as well as the respective controls the fresh weight increased with the age of the plant up to the stage when leaf fall began in the plant. The results are again in accordance with the earlier findings on this plant (Parashar et al., 2011; Pholphana et al., 2013). Accession V₃ yet again displayed more vigorous growth than accession V₁ under all treatments.

5.2.3 Number of primary branches, secondary branches and 10th branch length

Addition of N led to significant increases in the number of branches, primary as well as secondary, in the plants of both the accessions. Such results have also been noted in rice (Chaturvedi, 2006), Araucaria angustifolia (Garbin and Dillenburg, 2008) and tomato (Zuraiqi and Battikhi, 1992). N is known to fuel fast foliage by creating bigger structures (taller plants) with larger surface areas for branching and foliage (Chaturvedi, 2006; Rodriguez, 2014). A high sensitivity of branching towards availability of N has been reported in several other studies as well (Fallahi and Khajeh-Hosseini, 2011; Zhou et al., 2011). The effect of N application on the lengths of 10th branch of the two accessions of Kalmegh at different growth stages was non-significant at lower levels of N. All the three parameters under discussion significantly increased with the age of the plant in both the accessions. Accession V₃ had higher number of primary and secondary branches as well as longer 10th branches than accession V₁ in the control and different treatments of N proving it to be a high yielding accession. S addition also led to significant increase in the number of primary branches and the number of secondary branches in the plant at all treatments and plant growth stages. These findings are confirmed by several earlier reports in different plants like faba bean (Abdelhamid et al., 2013), coriander (Meena, 2015), onion (Mishu et al., 2013), corn (Manesh et al., 2013), and rapeseed (Ahmad et al., 2005) who also noted increased growth and branching with sulfur addition in the plants. In our study we also noted that S
application increased the lengths of the 10th branch in both the accessions but the increase was not significant at lower doses of applied sulfur. Several earlier studies have also noted increase in plant biomass through improvement in overall growth with sulfur application (Abd and Mona, 2013; Jamal et al., 2005; Mazher et al., 2006). The increase in growth and branching with sulfur application could be imputed to a higher absorbance of micro nutrients like P, Fe and Zn (Khodadad, 2012) or due to its interaction with N availability there by affecting the management of the two nutrients and consequently the growth of the plants as already discussed. We also noted that branching in the plants of both the accessions increased with the age of the plants in control as well as all treatments of sulfur. Significant increases in the number of branches both primary and secondary were observed with the addition of 24-epibrassinolide in both the accessions of the plant. The outcome is in accord with the results obtained by Akram et al. (2014) which reported an increase in the number of branches in *Jasminum sambac* with the application of 24-epibrassinolide and Eskandari and Eskandari (2013) and Hagh highi et al. (2014) which reported increased growth in *Satureja khuzestanica* and wheat with brassinolides application. Brassinolides affect cell division, elongation, and differentiation (Kang and Guo, 2011). The physiological pathways by which brassinolides control such processes include their effects on carbohydrate allocation and assimilation, and cell wall properties modification besides others. Apparently brassinolides interact with other plant growth regulators thereby coordinating different growth processes. On the molecular level brassinolides affected growth is controlled through the up-and down-regulation of several genes (Kang and Guo, 2011). Components like BZR1 and BES1 which positively regulate brassinolides responses and the commonly acknowledged downstream elements like EXO regulate brassinolides responsive genes and growth promotion (Müssig, 2005). Brassinolides have also been associated with the transfer of sugar from leaves to the growing tips (Nakajima and Toyama, 1995; Nakajima and Toyama, 1999). Such a response is important since accelerated growth increases the demand for carbohydrates in the growing region for biosynthetic metabolism. In our study we also noted that epibrassinolide addition led to significant increase in the lengths of 10th branches in both the accessions of the plant. Branching in the plants continued with the age of the plant in both the control as well as treatments as expected. Ethylene addition also led to significant increase in the branching in both the accessions of the plant however, exogenous ethylene had no effect on the lengths of the 10th branch in either HYA or LYA. Such a response has also been reported in *Chrysanthemum cinerariaefolium* plants at low concentrations of ethylene (low
concentrations at par with the optimum concentrations used in our study) (Haque et al., 2007). Increase in the number of branches at lower concentrations of ethylene has also been reported in *Hydrilla verticillata* (Klain, 1986). Exogenous application of ethylene has also been reported to increase branching in different species of *Kalanchoe* like *K. marnieriana*, *K. fedtschenkoi*, *K. glaurescens*, and *K. royundifolia* (Currey and Erwin, 2012).

### 5.2.4 Total number of leaves, Mean leaf area and Bush width

N addition significantly increased the number of leaves in both HYA and LYA. Significant increase in mean leaf area was also observed with N addition. Increased leaf number and area along with increased branching under N application ensured significant increase in bush width in both the accessions as well. The exaggerated leaf volume per plant with N addition is possibly explained by the fact that N increases plant growth and height through increase in the number of nodes and internodes, subsequently leading to greater leaf production in the plant. Production of greater number of nodes under N application leading to increase in leaf volume has also been advocated by Okajima et al. (1983), (Jones et al., 1995) and (Sawi, 1993). Amin (2011), Aminzadeh and Namazari (2013), Abbasi et al. (2013) and (Gungula et al., 2005) also reported a significant increase in number of leaves and leaf area in maize plants. Significant increase in leaf area with N addition is also on record (Boon et al., 2005). Increase in leaf area could have been due to the fact that N influences leaf expansion and development through it regulation of growth in plants (Amin, 2011). These results are in accord with the findings of Watson (1952) and El-Noemani et al. (1990) who reported that N significantly increased leaf area through its effect on leaf elongation. HYA had higher number of leaves than LYA under all treatments of N. Addition of S also led to significant increase in the number of leaves and leaf area in both the accessions of the plant. Increase in the leaf biomass with sulfur application has also been reported in several earlier studies as well (Channabasamma et al., 2013; Farooqui et al., 2009; Mazher et al., 2006; Wang, 2007). Sharifi (2012) observed a significant increase in leaf area in brassica plants with sulfur addition. An increase in leaf area with sulfur addition has also been observed in other plants like *Dalbergia sissoo* (Mazher et al., 2006), *Melissa officinalis* (Afkhami-Fathabad et al., 2014), mustard (Rais et al., 2013) and sunflower (Goutam, 2012). The increase in the foliage of the two accessions could be attributed to improved growth with sulfur application and the effect of sulfur application on the photosynthesizing tissues of the plants (Sharifi, 2012). The increase in the number of leaves, number of branches and leaf area also ensured that sulfur
addition also led to significant increases in the bush widths of both the accessions of the plant. Application of 24-epibrassinolide led to a significant increase in leaf number and area, in both the accessions, in a dose dependent manner. In addition epibrassinolide application also lead to significantly longer 10th branches in the plants of both the accessions of the plant. Similar results have also been reported by Mahesh and Rao (2014) in Ashwagandha, Shahid et al. (2015) in pea and Swamy and Rao (2011) in Coleus. The results are also consistent with the studies of Fariduddin et al. (2013) who noted significant increase in leaf area and number of leaves with epibrassinolide application in Cucumis sativus. The increase in leaf area and number of leaves with epibrassinolide application can be imputed to the physiological role of epibrassinolide in increasing photosynthetic area to produce more carbohydrate for plant growth (Nakajima and Toyama, 1995; Nakajima and Toyama, 1999). HYA produce higher number of leaves with greater surface area than LYA under all treatments of epibrassinolide. This can be ascribed to the differences in framework and cultural operations of the two accessions. In our study we also noted that ethylene addition led to significant increase in the number of leaves per plant and leaf area in both the accessions of the plant. Our results are in accordance with the several earlier studies (Iqbal et al., 2012; Iqbal et al., 2011; Singh et al., 2010). Currey and Erwin (2012) also reported a significant increase in the volume of leaves and leaf area in different species of Kalanchoe with ethylene application.

5.3 Physiological and Biochemical characteristics

5.3.1 Chlorophyll and soluble protein content

Soluble protein content and chlorophyll (a, b and total) content witnessed a significant increase with N addition in both HYA and LYA. Maximum Protein content was observed at the pre-flowering stage and it gradually decreased with the age of the plant suggesting an increased need for protein synthesis during the early growth of plants. The increase in chlorophyll and protein contents in the plants with N addition is understandable, because N is an integral part of proteins and chlorophyll molecules and therefore affects protein formation, chloroplast formation and the accumulation of chlorophylls in them (Daughtry et al., 2000; Tucker, 2004). Increase in leaf chlorophyll and protein content with N addition has been reported in several studies from time to time (Bojović and Marković, 2009; Hokmalipour and Darbandi, 2011; Kumari, 2011; Zhang et al., 2013). Ványiné et al. (2012) also observed a significant increase in soluble protein and leaf chlorophyll contents in maize with N addition.
Addition of S also led to significant increases in the leaf soluble protein contents and chlorophyll contents (a, b and total) in both the accessions. Several earlier studies have also reported similar findings (Dilmaghani et al., 2010; Mostafa and Abd El-Kader, 2006; Pagani and Echeverría, 2012). S is an important component of amino acids such as cysteine and methionine which are important constituents of proteins. An increase in s availability would therefore mean a greater production of these two amino acids and consequently higher protein content (Koprivova et al., 2008). Sulfur might have increased chlorophyll contents by improving N uptake and plant metabolism (Mostafa and Abd El-Kader, 2006; Salvagiotti et al., 2009). S being an important component of ferredoxins, (Fe-S protein in the chloroplasts) is therefore also important for the synthesis of chlorophyll (Havlin et al., 2005). Exogenous application of 24-Epibrassinolide also improved the leaf chlorophyll content and soluble protein content in both the accessions. Similar observations have been made in geranium (Swamy and Rao, 2009) and coleus (Swamy and Rao, 2011). Epibrassinolide induced high levels of chlorophyll may be attributed to increase in carbohydrate levels under epibrassinolide application. Several studies are present which report high protein and chlorophyll content in plants treated with brassinosteroids (Swamy and Rao, 2008). In Chlorella vulgaris brassinolides application has been reported to enhance the levels of chlorophylls (Bajguz and Czerpak, 1998). A significant increase in protein contents following epibrassinolide application has also been reported in mustard (Arora et al., 2010b). We also noted a significant increase in chlorophyll and protein contents in a dose dependent manner with ethylene application in both the accessions of our plant. Such a response has also been observed in earlier studies (Iqbal et al., 2012; Iqbal et al., 2011; Khan, 2004a; Singh et al., 2010). Ethylene influenced alterations in the efficiency of ribulose-1,5-bisphosphate carboxylase and carboxylation efficiency have been reported to be the primary factors behind ethylene the ethylene induced increase in photosynthesis. An indirect influence of ethylene on stomatal aperture is also considered a factor for ethylene stimulated photosynthesis (Iqbal et al., 2012; Iqbal et al., 2011). Khan (2004b) have demonstrated a significant positive correlation between 1-aminocyclopropane carboxylic acid synthase (enzyme which limits the rate of ethylene biosynthesis), and photosynthesis of mustard cultivars.

5.3.2 N metabolism

Increased rates of N and S fertilizers led to significant increase in NRA in plants, but in case of N after a dose of 120 kg ha\(^{-1}\) there was either a non-significant or significant decrease in
the NR activity. The decrease at the higher N treatment might have occurred because of an upper limit to resource utilization and accordingly plant performance (Nejidat et al., 1997). There are also limits for the levels of all the enzymes of N metabolism that a plant can accommodate. Our results showed that NR activity increased significantly with N addition in correspondence with several earlier studies (Hasaneen et al., 2008; Zhang and Li, 2007; Zhang et al., 2009). Both the accessions differed significantly in NR activity at all treatments of applied fertilizer. Accession V_3 had high NR activities than accession V_1 in controls as well as at all treatments. Differences in the NR activities between different accessions and genotypes has been reported earlier as well (Harada et al., 2003). NR activity is influenced by factors like temperature (Harris and Whittington, 1983), light intensity (Anjana et al., 2006; Lillo, 1994), and nitrate supply (Li and Oaks, 1993) besides different seasons, tissues, and plant growth stages. But in our study most of these factors were common for both the accessions and therefore must not have contributed any significant effect on the genotypic comparison of NR activities in the two accessions. The existent differences in the NR activities of the two accessions therefore represent the actual genotypic differences between the two. The conversion of NO_3^- to NO_2^- catalyzed by NR is considered as the rate limiting step nitrate-N conversion to amino acids and consequently proteins (Ferrario-Méry et al., 1998). NR being an inducible enzyme (substrate), its mechanism of regulation is complex and is affected by several direct and indirect factors. Plants sense their internal N status and N availability in the environment through nitrate sensing (Stitt, 1999). Nitrate sensing at a cellular level, leads to reprogramming of metabolism thereby allowing nitrate assimilation and its consequent incorporation into amino acids and other organic compounds. At the level of a whole plant, nitrate sensing regulates apportioning and development allowing nutrient uptake, root growth and variations in the availability of nitrate with time (Stitt, 1999). Nitrate itself may serve as the signal or the signals may come from products of nitrate assimilation or even more directly in response to alterations in other cell constituents or growth rate. A negative feedback mechanism on nitrate acquisition originating from downstream events may also be operative. In a particular state of affairs, one of the signals or a combination of signals may interact to invoke a particular response. Enzymes of GOGAT pathway (Ammonia assimilation) and the genes encoding uptake systems of nitrate (low and high-affinity), for NR (NIA) and NiR (NII) are induced by nitrate. The increase in transcript results in enhancement of uptake rate of nitrate, enhancement in the quantity and activity of NIA protein and a parallel gain in the NII and glutamine synthetase activity. The synthesis of α-
oxoglutarate and other organic acids is also essential for nitrate assimilation because in the GOGAT pathway α-oxoglutarate acts as an acceptor for NH₄. Nitrate assimilation also requires the synthesis of malate which substitutes for nitrate by acting as a counter anion, to preclude alkalinisation. Therefore genes for redox buffers like malate and C-acceptors like α-oxoglutarate and other organic acids are also induced by nitrate. Nitrate also induces genes for synthesizing redox equivalents required during respiration. Nitrate therefore leads to widespread and rapid changes in the quantity of different transcripts controlling expression of several enzymes of C and N metabolism. Regulation of C and N metabolism facilitates nitrate metabolism and its incorporation into amino acids. A concurrent increase in the activity of enzymes of N metabolism with N addition in both the high NR (HYA) and low NR (LYA) accessions as was observed in our study substantiates the fact that all the enzymes involved in N metabolism work in a well-controlled and coordinated manner in order to assimilate N in the plants. Therefore N addition led to significant and concurrent increase in the enzymes of nitrate and ammonium assimilation in the two accessions.

Addition of S also led to significant increases in the activity of all N metabolizing enzymes in both the accessions of the plant. Similar results have also been reported in studies from time to time (Ahmad et al., 2007; Astolfi et al., 2004; Siddiqui et al., 2012). As already discussed S requirements in plants are closely related to plant growth and N availability (Ahmad et al., 2007; Coleman, 1966; Hawkesford, 2007). S is also an important component of amino acids cysteine and methionine, which are important constituents of proteins, cofactors and secondary products (Koprivova et al., 2008). S containing thiol groups are used in several redox reactions in biological systems (Nazar et al., 2011). The relationship of S with N are well documented (Siddiqui et al., 2012) since sulfate has been reported as a transporter of NO₃⁻ in vacuoles (Miller et al., 2009). Therefore S might improve N metabolism in the plants by improving N uptake since NR is a substrate inducible enzyme (Abdallah et al., 2010; Salvagiotti et al., 2009) or through its effect on protein formation.

Application of plant growth regulator 24-epibrassinolide also led to significant and dose dependent increase in the activities of N metabolizing enzymes in both the accessions of the plant. Genotypic differences in the two accessions ensured that HYA had higher enzyme activities in control as well as treatments than LYA at all plant growth stages. It was also noted that the enzyme activities declined with the age of the plant. Similar results depicting an increase N metabolizing enzymes with epibrassinolide application has been reported.
earlier as well under both stressful and optimal conditions (Anuradha and Rao, 2001; Dalio et al., 2013; Hayat et al., 2007). It is argued that epibrassinolide addition increases nitrate uptake in plants (Dalio et al., 2013) which may thereof have increased the NR activity because NR activity is induced by nitrate availability. Since all the enzymes of N metabolism operate coordinately it is possible that an increase in NR activity, which results in the conversion of nitrate into nitrite, that forms the rate limiting step in N metabolism, would have also resulted in an increase in the activity of other N metabolizing enzymes. brassinosteroids (Homobrassinolide and 24-Epibrassinolide) have also been reported to improve NR activity in tomato (Yadav et al., 2012) and pea (Shahid et al., 2011). The epibrassinolide response might have been due to the effect of brassinolides on translation and (or) transcription of the NR genes (Khripach et al., 1999). Ethylene application did not affect the activity of N metabolizing enzymes in any of the two accessions however a significant increase in NR activity was observed at higher doses of ethylene (150 and 200 µl L⁻¹) in both HYA and LYA at the pre-flowering stage. No effect of ethylene application was observed on N metabolism of the plants at the flowering and post-flowering stages. Lone et al. (2010) also reported significant increase in NR activity with ethylene addition in mustard. Khan et al. (2008) also observed a significant increase in NR activity in with low doses (low doses in the study were equivalent to our highest dose) of ethylene in mustard plants. A link between N availability and ethylene evolution has also been put forward which might have been how ethylene affected NR activity in plants at the pre-flowering stage (Khan et al., 2008). Genotypic differences in the activities of N metabolizing enzymes between the two accessions were persistent even under ethylene application.

5.4 Quality parameters

5.4.1 Active constituents

A. paniculata derives its economic importance from the highly valued diterpene lactones present in the plant. All parts of this plant are rich in these terpene lactones but leaves have been reported to contain the maximum amount of these lactones. The amount of these lactones varies with the plant growth stage and optimum amounts of these lactones have been reported to be present at the flowering stage (Sareer et al., 2012; Sareer et al., 2014). Total lactone content and AP1 increased with all treatments of nutrients and plant growth regulators. Flavonoid contents in the plants witnessed an increase only under the treatments
of plant growth regulators, while as the phenolic contents increased under the treatments of sulfur and ethylene. AP2 content increased at lower levels of applied N after which there was no further increase in the AP2 content in the HYA. AP2 contents in the LYA did not vary with N addition. Under S and ethylene treatments AP2 contents increased significantly at the higher treatments only in the LYA while they increased significantly in a dose dependent manner in the HYA. AP2 content did not vary with N and ethylene treatments in any of the two accessions, while as addition of S and epibrassinolide invoked a significant positive response in the concentrations of the compound in both the accessions. A significant increase in AP4 content was observed under the treatment of 24-epibrassinolide and N, but in case of N there was no significant increase in the concentration of the compound beyond the 120 kg ha\(^{-1}\) level. Addition of ethylene did not have any effect on the AP4 contents in any of the two accessions. S addition led to significant increase in the AP4 contents of HYA while the increases in the LYA were significant only at the higher treatment of S.

Increase in the yield of active principles with N addition has been noted in several medicinal plants from time to time (Mishra and Jain, 2013; Tiwari et al., 2012; Verma et al., 2010; Wang et al., 2008). No significant effect of N application on the flavonoid and phenolic contents as was observed in our study is also on record (Wang et al., 2008). N addition has also been reported to increase essential oil content in fennel (Ayub et al., 2011). The increase in secondary metabolite concentration with N addition could be possible because N has a strong effect on the basic metabolism which may be directly or indirectly influence secondary metabolite production (Baricevic and Zupancic, 2002). The changes in the concentration of the secondary metabolites with plant age and their differential response to N fertilization can be possible because the secondary metabolites keep switching between alternate forms depending on the needs of the plant. Several studies have also reported the positive effect of S application on secondary metabolites in medicinal plants as was noted in our studies (Ahmad et al., 2007; Jamal et al., 2005; Omirou et al., 2009; Tunçtürk et al., 2011). Both the plant growth regulators 24-epibrassinolide and ethylene have been reported to significantly alter the secondary metabolites in medicinal and other plants. Swamy and Rao (2011) and Swamy and Rao (2008) reported significant increase in active principles of coleus and geranium respectively following brassinosteroids application. Several other studies also have reported the positive effects of brassinosteroids application on the secondary metabolites of different medicinal and aromatic plants (Bajguz and Piotrowska-Niczyporuk, 2013; Xu et al., 2014). Ethylene has also been demonstrated to significantly improve the content of different
secondary metabolites in plants as was found in our studies (Diarra et al., 2013; Linden et al., 2001; Sharafzadeh, 2012). Our results on ethylene treatment are also consistent with the studies of Shibli et al. (1997) who reported ethylene to significantly improve secondary metabolite production in Vaccinium pahalae.

5.4.2 GCMS profile of leaf extracts

No evident pattern was observed in the GCMS profiles of the leaf extracts under any of the treatments. However application of nutrients and plant growth regulators led to increase in the percentages of andrographolides in the leaf extracts. Differences in the percentages of individual compounds were noted under different application rates of treatments and between different treatments as well. Some new compounds were synthesized in the plants in response to applied treatments while the production of some others was down-regulated. Percentages of some compounds increased at lower level of a treatment and then decreased at a higher level and vice versa. Similar results have also been noted with different nutrient and plant growth regulator applications earlier as well (Kelly, 2013; Urbanczyk-Wochniak and Fernie, 2005).

5.5 NUE parameters

Nitrogen addition led to significant increases in WPNC and WPNN in both the accessions. This may have occurred due to poor N utilization and its subsequent accumulation in the plants (Jamaati-e-Somarin et al., 2008; Wang et al., 2014). Differences were observed between the two accessions in their relative WPNC and WPNN. LYA maintained higher N contents in plants than HYA in control as well as at all treatments of N. Low N contents in higher biomass plants could be due to the reason that limited amounts of N amounts get diluted by larger mass of carbohydrates (Kibite and Evans, 1984). Grain yield increased significantly with N addition in both the accessions however HYA had higher grain yields that LYA at all treatments of N. Jamaati-e-Somarin et al. (2010a) have also reported a significant increase in wheat grain content with N addition. Significant increase in GNC and GNN were observed with low doses of N in LYA while GNC and GNN increased in a dose dependent manner in the HYA. The differences might have been because of poor translocation of N into grain in the LYA at higher N concentrations (Anjana, 2006). Differences in the translocation efficiencies in the two accessions are determinants of genotypic differences between the two accessions (Anjana, 2006). WPNC and WPNN
showed a significant positive correlation with grain yields and plant biomass in both the accessions under N impoverishment. Similar conclusions have also been arrived at in wheat plants (Desai and Bhatia, 1978; Van Sanford and MacKown, 1986). Nitrogen utilization efficiencies both biomass and grain witnessed a significant decrease with N impoverishment in both the accessions. HYA however maintained higher NutE1 and NutE2 at all N treatments than accession V1. A decrease in plant NUE with N addition has been observed in earlier studies as well (Jamaati-e-Somarin et al., 2008; Jamaati-e-Somarin et al., 2010b; Zand et al., 2014). A significant decrease in NTE, HI and GY/GN was also observed with different treatments of N however, decrease in NTE at lower levels of N were not significant. Decrease in NUE with N addition might have resulted in poor translocation efficiencies and consequently lesser grain yield per unit of grain N. Decreases in NTE and GY/GN with N addition have also been observed in earlier studies (Jamaati-e-Somarin et al., 2008; Jamaati-e-Somarin et al., 2010a; Jamaati-e-Somarin et al., 2010b).

Sulfur application significantly decreased whole plant nitrogen concentration in both the accessions. Sulfur is believed to increase N uptake (Salvagiotti et al., 2009) and therefore the decrease in N concentrations in the plant may have been due to higher increase in biomass diluting the N concentrations in the plants (Kibite and Evans, 1984). An insignificant decrease in N concentration in the shoots of Tef following S application has also been reported under N deficient conditions (Habtegebrial and Singh, 2006). Another possible reason for the decrease in N concentrations might be due to the translocation of available N into the grain as is evidenced by higher GNC, GNN and grain yields in S treated plants. Increase in grain yield and grain N concentrations with S application has been demonstrated in several other plants as well (Girma et al., 2005; Potarzycki and Grzebisz, 2007). S has also been demonstrated to increase the photosynthetic assimilation of N in plants which results in improved grain yields and dry matter. Nitrogen utilization efficiencies both biomass and grain increased significantly with S addition in both the accessions which is in accordance with the findings of Habtegebrial and Singh (2006) who reported significant improvement in NUE in Tef with S application. This might be because of the involvement of S in protein biosynthesis, there by determining yields. Schnug et al. (1993) have also reported significant increases in fertilizer NUE with S addition for mustard and wheat crops. S addition did not have any effect on the %N losses in any of the two accessions of the plant. A significant increase in nitrogen translocation efficiency and harvest index was also observed in our study which is consistent with earlier studies as well (Namvar and Khandan, 2014; Zuzhang et al.,
2010). The effect of 24-epibrassinolide and ethylene application was similar to the effect of S application except that 24-epibrassinolide had no significant effect on the whole plant N concentrations. Grain N utilization efficiencies increased significantly only under higher treatments of epibrassinolide. The positive effect of ethylene on NUE could have been due to its effect on N uptake and photosynthesis in the plants (Shahid et al., 2011). Improved N uptake (Janeczko et al., 2010) and enhanced enzyme activities of N metabolism (Yuan et al., 2012) by epibrassinolide application resulted in better growth of the plants and consequently better indices for different parameters of NUE. Ethylene has also been demonstrated to improve photosynthetic NUE in mustard plants (Khan et al., 2008).

It will now suffice to say that both nutrients and plant growth regulators used in the present study have the potential for significantly improving yields in the plants. Losses through cultivation of low yielding accessions can be minimized by a careful selection process and the subsequent nutrient and plant growth regulator application on the plants. Environmental effects of N can be minimized by incorporating other nutrients like S (used in our study) and K (reported in earlier studies) and plant growth regulators like ethylene and epibrassinolide that were used in our study.