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Original Article

Novel NARC-G1 garlic: comparative allicin quantification with morpho-biochemical & genetic profiling

O raro NARC-G1 alho: quantificação comparativa de alicina com perfil morfo-bioquímico e genético

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Abstract

Garlic (*Allium sativum*) is an important cash food crop, and the biotechnology industry has considerable interest in the plant because of its medicinal importance. These medicinal properties are attributed to organosulphur compounds as the accumulation of these compounds varies according to genotype, locality, light quality, and cultivation practices. In this study, we compared a newly developed garlic variety NARC-G1 by National Agricultural Research Centre (NARC), Islamabad, Pakistan with three different garlic cultivars and highlighted the distinctive attributes like phenotypic characteristics, the content of allicin, elemental profile, and gene polymorphism. Phenotypic analysis showed NARC-G1 has significantly higher bulb weight (66.36g ± 18.58), single clove weight (5.87g ± 1.041), and clove width (17.41mm ± 0.95) which directly correlates to the size of the garlic. The analytical analysis showed the highest allicin content (4.82 ± 0.001) in NARC-G1. Genotyping of the alliinase in all four cultivars showed indels in the gene resulting in distinguishable changes in organosulphur compounds' profile. NARC-G1 is unique from other garlic cultivars and could be the best choice for mass production with proper cultivation and irrigation management. Moreover, for Pakistan NARC-G1 could be a potential contender to earn the industrial benefits with inland cultivation instead of importing garlic alleviating the economic burden.

Keywords: garlic (*Allium sativum*), alliinase gene, NARC-G1 garlic, ICP-OES, HPLC-based allicin quantification.

Resumo

O alho (*Allium sativum*) é uma importante cultura alimentar de rendimento e a indústria biotecnológica tem um interesse considerável na planta devido à sua importância medicinal. As propriedades medicinais são atribuídas aos compostos organossulfurados, enquanto o acúmulo de sulfóxidos de cisteína (CSOs) varia de acordo com genótipo, localidade, qualidade da luz e práticas de cultivo. Neste estudo, comparamos uma variedade de alho recém-desenvolvida NARC-G1 pelo Centro Nacional de Pesquisa Agropecuária (NARC), Islamabade, Paquistão, com três diferentes cultivares de alho, e destacamos os atributos distintivos, como características fenotípicas, conteúdo de alicina, perfil elementar e gene polimorfismo. A análise fenotípica mostrou que o NARC-G1 tem peso de bulbo significativamente maior (66.36g ± 18.58), peso de um único dente (5.87g ± 1.041) e largura do dente (17.41mm ± 0.95), o que se correlaciona diretamente com o tamanho do alho. A análise analítica indicou maior teor de alicina (4.82 ± 0.001) no NARC-G1. A genotipagem do gene da aliinase em todas as quatro cultivares mostrou indels no gene, resultando em mudanças distinguíveis no perfil das CSOs. O NARC-G1 é único em relação a outras cultivares de alho e pode ser a melhor escolha para produção em massa com cultivo adequado e manejo de irrigação. Além disso, para o Paquistão, o NARC-G1 pode ser um potencial candidato para obter os benefícios industriais com o cultivo no interior, em vez de importar alho, aliviando o ônus econômico.

Palavras-chave: alho (*Allium sativum*), gene da aliinase, alho NARC-G1, ICP-OES, quantificação de alicina baseada em HPLC.

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1. Introduction

Garlic (*Allium sativum*) is an aromatic herbaceous plant that belongs to the family Alliaceae and is the most ancient, cultivated plant that originated for the first time in Central Asia and is now cultivated throughout the world for economic, pharmaceutical, and nutritional purposes. Garlic is rich in volatile and non-volatile compounds which play a role in bioactive and therapeutic activities serving as an antioxidant, anti-inflammatory, antibacterial, antifungal, and immunomodulatory (Amagase et al., 2001; Mikaili et al., 2013). Due to unique flavor, aroma, and diverse nutritional properties, garlic is widely used in cuisines across the globe (FAO, 2015). Due to the known diverse health benefits of garlic, studies had been invested in exploring the role of active phytochemicals (Nazir and Chauhan, 2019; Petropoulos et al., 2018). Phytochemical analysis showed the presence of various bioactive metabolites of diverse compositions such as saponin, tannin, carbohydrates, cardio glycoside, alkaloids, flavonoid, glycoside, and organosulfur compounds encompassing diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, S-allyl-cysteine (SAC), and S-allyl-cysteine sulfoxide (alliin) (Amagase, 2006; Boonpeng et al., 2014; Mikail, 2009; Percival, 2016; Ried and Fakler, 2014; Schäfer and Kaschula, 2014; Seckiner et al., 2014). Amongst all allicin, one of the most important bioactive compounds, is derived in considerable amounts from alliin. For humans, allicin possesses remarkable therapeutic role, however the production of allicin in garlic is the result of defense response to the external harmful stimuli such as crushing, cutting, grinding, and heating (Liu et al., 2017). Allicin is a volatile compound and responsible for the characteristic pungent smell as well as anti-microbial, antifungal, anticancer, antiatherogenic, and antioxidant properties (Fry et al., 2005; Leontiev et al., 2018; Rabinkov et al., 1998). Type and concentration of bioactive compounds extracted from garlic depend on a set of parameters including the cultivar, phenotypic traits, stage of maturation, location, and genetic variability. Considering these factors, comparative phenotypic and phytochemical screening of various garlic cultivars explored for the variable presence of bioactive compounds, mineral content, and macromolecules (Prati et al., 2014).

In multiple studies, the allicin content were quantified using HPLC in geographically and genetically different garlic cultivars suggesting the cultivation pattern and geographical distribution changes the genetic basis of garlic varieties, affecting the bioactive content (Ipek et al., 2015; Kumar et al., 2019; Yoo et al., 2010). Since the enzymatic reaction of the alliinase produces the bioactive compound in garlic, the studies analyzed the genetic profile of the gene. These studies revealed the existence of multiple variations i.e., insertion/ deletion in the exonic and intronic regions which play the significant role in differential expression of the *alliinase* gene hence responsible for the allicin content in individual garlic genotypes (Ovesná et al., 2015; Sayadi et al., 2020).

In Pakistan, National Agricultural Research Centre (NARC) Islamabad developed a new garlic variety NARC-G1. This garlic variety has extraordinary yield and phenotypic traits in comparison to other locally cultivated varieties, for instance, Italian garlic, Desi Gulabi garlic, and Chinese garlic thus making NARC-G1 garlic a superior candidate for the agriculture and industrial sector. Larger bulb size, better plant height, and vigorous compatibility with harsh environment makes NARC-G1 candidate of interest for national and international agricultural benefit (Khan et al., 2018). The new variety NARC-G1, is under investigation not only for mass production but also to gain pharmaceutical and therapeutic benefits. Previously very few studies investigated the phenotypic traits of NARC-G1 however, genetic basis for the production and content of phytochemicals in NARC- G1 in comparison with the other breeds of garlic cultivated in the region remain elusive to date. In our novel study, we explored the differential presence of bioactive compound allicin between NARC-G1 and other garlic varieties utilizing HPLC and conducted the elemental profiling of each garlic variety. Furthermore, we have explored the genotypic variation of the *alliinase* gene among all the garlic cultivars. Through this novel work, we have found the unique attributes of NARC-G1 garlic and shed light on how these traits can make it an exceptional and best choice for agriculture and industrial use.

2. Materials and Methods

2.1. Plant cultivation for phenotypic analysis

Four garlic varieties: Italian, Desi Gulabi, Chinese, and NARC-G1 were cultivated over Three seasons from 2018 to 2020 during September at Bioresources Conservation Institute, NARC Islamabad (33.67, 73.13) with a plant-to-plant distance of 8 cm with distal end upward. The experiment was laid in five replications using randomized complete block design with a random selection of 5 bulbs for each garlic cultivars (Khan, 2021). All the planted cultivars were harvested in April of following year and the parameters for morphological analysis were recorded.

2.2. Elemental analysis through ICP-OES

All reagents, diluents, and eluents were prepared using type-1 water having 18 MΩ.cm resistivities from the Millipore water system (Milli-Q® Merck KGaA, Darmstadt, Germany). Nitric acid (65% (v/v) obtained from Merck (Merck KGaA, Darmstadt, Germany) while multi-element stock solution having all the analyzed elements bought from Sigma-Aldrich, USA.

2.2.1. Sample preparation

The samples were analyzed in triplicate employing ICP-OES (Avio 500, Perkin Elmer, USA). The cloves from different garlic bulbs were sliced and dried in a Büchi TO-50 (Büchi, Switzerland) drying tube at 65 °C under moderate vacuum until constant weight and successively ground. After grinding, 0.1 g of garlic powder was introduced into a Teflon digestion vessel together with 10 mL of nitric

acid and 10 mL of Type-1 water. The mineralization was conducted in a Multiwave Eco microwave oven (Anton Paar GmbH, Anton-Paar-Strasse Austria.) with Rotor vessels (HVT50-T16) at constant power (1000 W). The sample temperature was increased to 200 °C in 10 min and was kept at 200 °C for 40 min. The digested samples, cooled at room temperature, were transferred to a volumetric flask, and diluted to 50 mL with type-1 water. For the detection of analytes following parameters were employed; the plasma gas (14 L min^{-1} argon (Ar)), auxiliary gas (0.8 L min^{-1} Ar), nebulizer (1.0 L min^{-1} Ar), RF power (1500 W), a peristaltic pump (1.5 mL min^{-1}), and axial viewing distance of 15 mm were controlled in the Syngistix ICP software. The wavelengths were examined simultaneously.

2.3. Detection of allicin through HPLC

2.3.1. Reagents and chemicals

All reagents and chemical for analysis were HPLC-grade. Allicin standard was prepared according to the procedure of Institute for Nutraceutical Advancement (INA) method 110.001 (Prati et al., 2014) using recycling preparative HPLC (Model LC908W; Japan Analytical Industry (JAI) Co., Ltd, Japan) with stationary phase octadecylsilane (ODS) 20 x 270 mm. For elution, 50:50 water to Methanol (Merck, Darmstadt, Germany) was used. The allicin content was determined on the spectrophotometer (Evolution 201 UV/Vis, Thermo Scientific, USA) using an extinction coefficient of 10.0 mg/mL at 220 nm. Propylparaben (99.6%, Sigma-Aldrich) was employed as certified reference standard.

2.3.2. Analytical HPLC system

Chromatography was performed by using a Waters® HPLC analytical liquid chromatography (Waters Corporation, 34 Maple Street Milford, USA) equipped with an Auto-Injector (Model 2707), Photodiode array (PDA) Detector (Model 2996), and Stationary phase: Waters® Symmetry C18 Column, 5 µm, 4.6 mm X 250 mm and Breeze 2 software used for data processing.

2.3.3. Preparative HPLC system

For Preparative separation, recycling preparative HPLC (Model LC908W, JAI, Japan), equipped with UV Detector 310 and RI Detector RI-5, 20 mm x 270 mm JAIGEL-ODS-L-80 C18 column with 2 mL/min flow rate was used.

2.3.4. Sample preparation

The outer skin of garlic cloves was peeled off and cloves were weighed. The 0.5 - 0.8 g of weighed cloves were crushed in a manual garlic press rinsing with the addition of 25 mL chilled type II water (5 °C) in a beaker quantitatively and stirred in magnetic stirrer adding extra 25 mL water keeping the temperature at 5°C. All samples were filtered through a 0.45 µm filter membrane for HPLC analysis.

2.3.5. Chromatographic conditions

All analysis were performed at room temperature (23 ± 2 °C) under isocratic conditions. The separation was conducted using a Waters® Symmetry C18 Column (5 µm, 4.6 mm x 250 mm column). The mobile phase consisted of methanol-water (50:50 v/v) which was degassed and filtered in a PVDF 0.45 µm, (HVLP04700 Durapore® 47 mm Millipore®, USA) membrane filter under negative pressure. The UV detection was conducted at 220 nm at flow rate of 1.0 mL/min. Propylparaben (10 µg/mL) was used as the internal standard.

2.4. Genotyping of alliinase gene

For genotyping of *alliinase* gene of NARC-G1 and other cultivars, DNA extraction was performed utilizing method describe in literature (Rogers and Bendich, 1994). Firstly, garlic cloves were washed with distilled water followed by washing with 70% ethanol (Sigma-Aldrich, USA). Garlic samples were dried and grounded mechanically. Out of ground samples, 100 g of plant material was collected in a microcentrifuge tube (CITOTEST, China). Cetyltrimethylammonium bromide (CTAB, Fischer Scientific, USA) was added with proteinase K (Thermo Scientific, USA) and mixed thoroughly using a vortex (VM-10 WiseMix®, Witeg Labortechnik, Germany). The sample was incubated for 10 minutes and centrifuged (Model No:144816, Sartorius, Sigma, USA). The supernatant was separated and RNAse A (Thermo Scientific, USA) was added, the sample was incubated for another 10 minutes at 60°C. After incubation Chloroform (Sigma-Aldrich, USA) was added followed by centrifugation. The upper phase transferred in a new tube containing isopropanol (Merck KGaA, Germany). The sample was centrifuged, and a DNA pellet was obtained. The DNA pellet was dissolved in T.E buffer. The DNA was stored at -20 °C. Extracted DNA is visualized on 0.8% agarose gel (bio-WORLD, USA).

The gene-specific primers were designed on *alliinase* gene (Accession No: KR270357.1 NCBI; additional data are given in Appendix S1). All the samples were subjected to PCR (T100 Thermal cycler, BIO-RAD, USA) and the PCR products of the *alliinase* gene were sequenced through Sanger sequencing. All the obtained sequences were aligned with reference sequence using Clustal OMEGA software, to check the variation (indels) among all the garlic cultivar for the *alliinase* gene.

2.5. Statistical analysis

For statistical analysis IBM SPSS Statistics for Windows, Version 25.0 was used. All the phenotypic parameter across separate groups were analyzed using *post-hoc* Tukey's test (Appendix S2). Furthermore, the results of elemental analysis were analyzed statistically for mean (Appendix S3). Standard error bars show the significance < 0.05.

3. Results

3.1. Phenotypic analysis

All the garlic cultivars planted were analyzed for the following phenotypic traits upon harvesting: plant height (cm), bulb thickness (mm), number of leaves per plant,

number of cloves per bulb, weight of single bulb (g), weight of single clove (g), and clove width (mm) (Figure 1).

The plant height among the cultivars showed a significant difference with Italian garlic having minimum height among the cultivars (57.0 ± 5.67). The difference between NARC G-1 and Chinese garlic plant height showed least difference (66.38 ± 4.12 and 68.0 ± 3.24 respectively). Chinese garlic showed significantly better results for bulb thickness (56.06 ± 2.39) and number of leaves per plant (11.40 ± 1.51) as compared to Italian and Desi Gulabi garlic cultivars. Number of cloves per bulb showed completely different results with NARC G-1 having least number of cloves per bulb (7.53 ± 0.65) as compared to Desi Gulabi garlic (16.60 ± 2.70), which had the highest. For weight of single bulb (66.36 ± 18.58), weight of single clove (5.87 ± 1.041), and clove width (17.41 ± 0.95) NARC G-1 was significantly superior to Chinese, Italian and Desi Gulabi garlic. The graphical representation of phenotypic analysis among cultivars is showed in Figure 2 while the results of all the traits are in Appendix S2.

3.2. Elemental analysis

Utilizing ICP-OES technique, elemental profiling at various wavelengths was performed. Among all the cultivars no traces of metals like Silver (Ag), Aluminum (Al), Boron (B) Barium (Ba), Chromium (Cr), Copper (Cu), Lithium (Li), Lead (Pb), Molybdenum (Mo), Antimony (Sb) and Titanium (Ti) were detected (Appendix S3). Highest Calcium (Ca) amount (3.36 µg/g) detected at wavelength of 318 nm in NARC-G1 while in Desi Gulabi garlic (1.17 µg/g), Italian garlic (0.21 µg/g) at the wavelength of 317 nm whereas no traces detected in Chinese garlic. Phosphorous (P) detected in all the cultivars at wavelength of 215 nm with least amount in NARC G-1 (3.68 µg/g). Iron (Fe) was only detected in NARC-G1 in good amount (1.35 µg/g). Potassium (K) and Magnesium (Mg) were detected in all the garlic cultivars at the wavelength of 766 nm with NARC G-1 garlic having 2.04 µg/g and 1.26 µg/g, respectively. Sodium (Na) present in all the cultivars except Italian garlic at the wavelength of 590 nm. Nickle found at wavelength of 232 nm in traces in NARC-G1 (0.05 µg/g) and Italian Garlic (0.08 µg/g). Traces of silicon (1.67 µg/g) were detected only in NARC-G1 at the wavelength of 252 nm. All the garlic cultivars hold traces of Cadmium (Cd), a toxic heavy metal, detected at a wavelength of 229 nm.

3.3. Detection of allicin through HPLC

The peak obtained was diagnosed by HPLC using a UV detector at a wavelength of 220 nm. The chromatogram

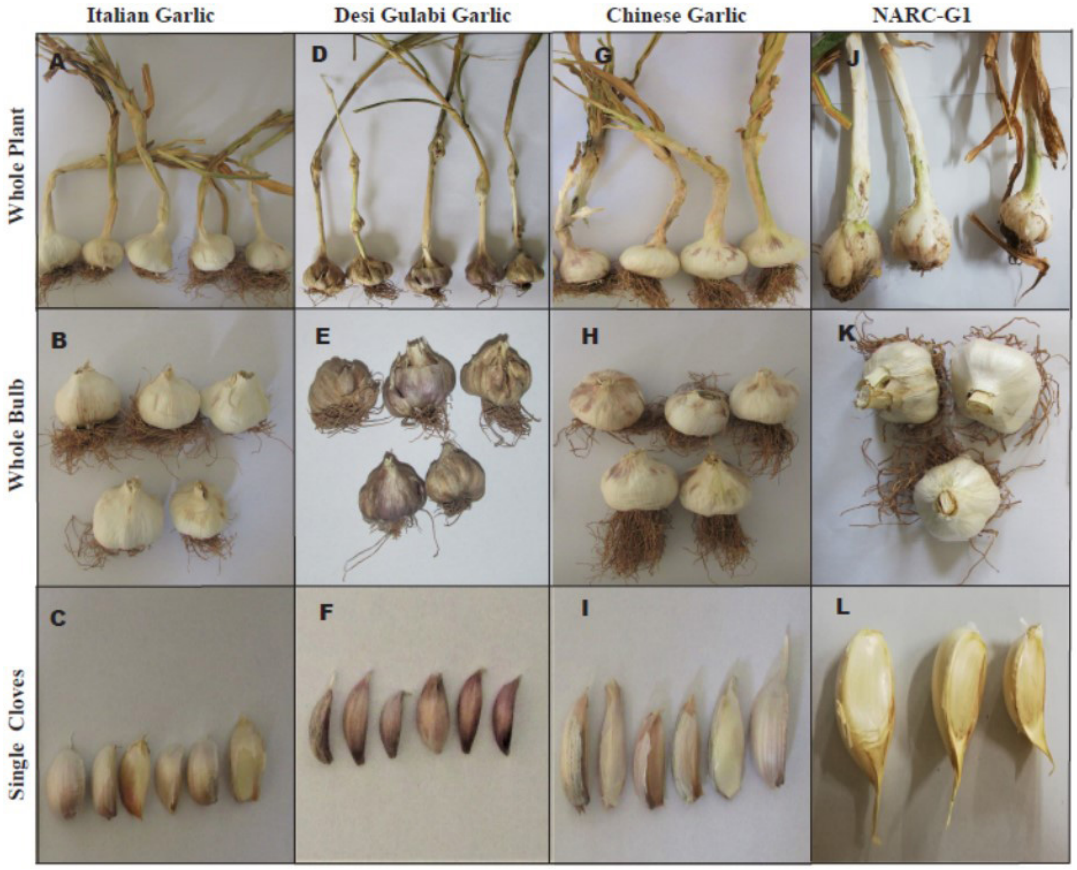

Figure 1. Phenotypic analysis of garlic cultivars A-C, D-F, G-I and J-L: show the representative images of Italian, Desi Gulabi, Chinese & NARC-G1: whole plant, whole bulb, single cloves, respectively.

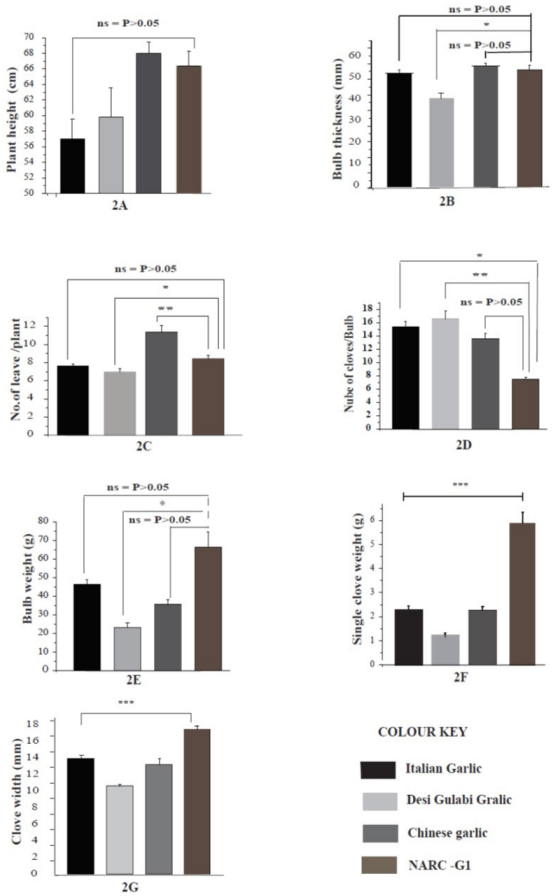

Figure 2. Significance level of phenotypic traits among garlic cultivars (Italian, Desi Gulabi, Chinese & NARC-G1) 2A: plant height of cultivars; 2B: bulb thickness; 2C: number of leaves per plant; 2D: number of cloves; 2E: weight of single bulb; 2F: single clove weight; 2G: clove width. (The ns=non-significant, *=significant difference, **= very significant difference, ***=highly significant).

obtained for the standard of allicin, and internal standard shown in Figure 3.

The concentration of allicin was measured in all the garlic cultivars using an equation of standard allicin curve. With retention time (5.25 min) the recovery of allicin obtained a maximum concentration of 4.82 ± 0.001 in NARC-G1 (Figure 4) while Desi Gulabi, Chinese and Italian garlic gave the concentration of 2.934 ± 0.001, 1.813 ± 0.011, and 0.011 ± 0.0006 (Table 1) respectively.

3.4. Genotyping of allicin

The *alliinase* gene was amplified through PCR using overlapping primers across the gene. The PCR products revealed variable sizes in all the selected cultivars (Figure 5). The PCR products were subjected to Sanger sequencing, followed by multiple sequence alignment (MSA), which revealed multiple indels in the *alliinase* gene of garlic cultivars (Table 2). The nucleotide sequences of all the garlic cultivars were submitted to GenBank, NCBI.

4. Discussion

This novel study investigated the allicin content as well as gene responsible for its production in the newly developed garlic variety, NARC-G1, in comparison with 3 garlic varieties (Italian, Desi Gulabi and Chinese) being commonly cultivated in Pakistan. Additionally, a comparative study encompassing morpho-biochemical profile i.e., phenotypic, and elemental analysis aimed to compare integral attributes among these garlic varieties.

As previously reported the geographical distribution impact the traits of an individual plant due to genetic variability (González et al., 2009; Mohammadi et al., 2014). Therefore, this comparative study of NARC-G1 garlic with Italian garlic, Desi Gulabi garlic and Chinese garlic extrapolated to understand the impact of geographical variation on NARC-G1. Sood et al. (2000) reported as

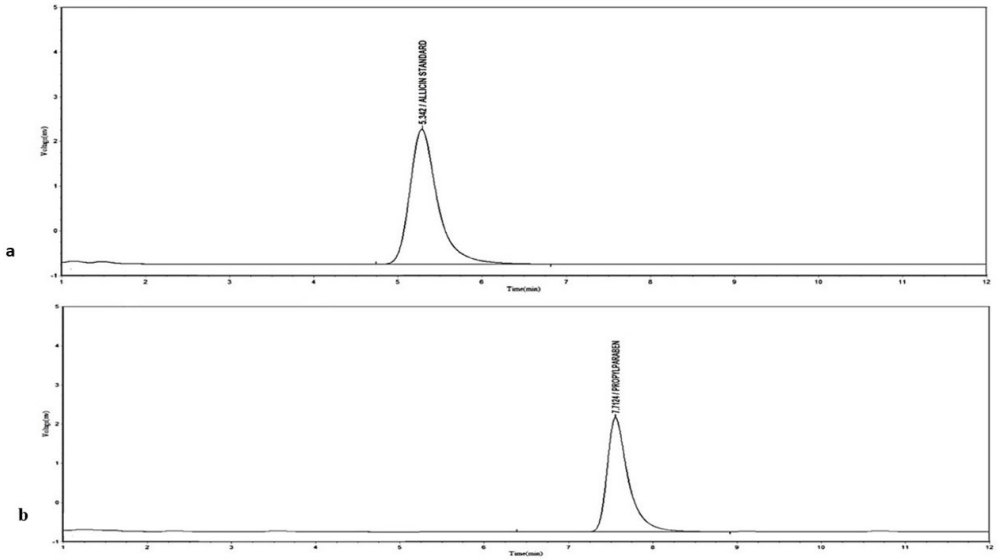

Figure 3. a: The chromatogram for standard allicin retention time 5.34; b: peak for internal standard Propylparaben retention time 7.712.

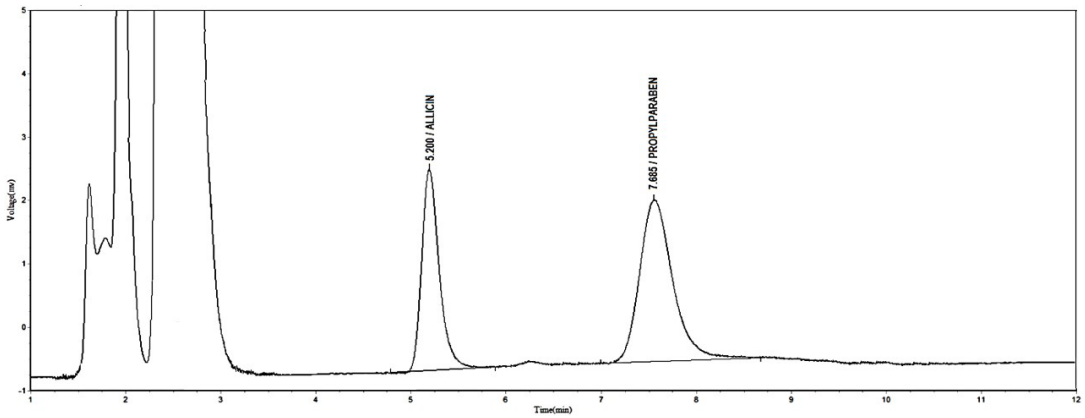

Figure 4. Chromatogram for allicin in NARC-G1garlic with internal standard.

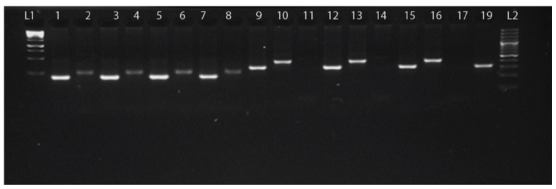

Figure 5. *Alliinase* gene amplification with overlapping primers across the gene on 2% gel L1= DNA 100 bp Ladder, L2= DNA 1 kb Ladder, amplified gene fragments from 1-19 with primer sets 1-4 (Appendix S1).

Table 1. Recovery of allicin from different cultivars and stages; recovery of allicin through HPLC in all the four garlic cultivars (Italian, Desi Gulabi, Chinese and NARC-G1) with retention time and mean of recovery concentration.

Garlic cultivars	Component	Retention Time (min)	Recovery (μg.mg $^{-1}$)
Italian	Allicin	5.23	0.0110
		5.28	0.0111
		5.31	0.0122
	Mean = 0.011 ± 0.0006		
Desi Gulabi	Allicin	5.32	2.921
		5.31	2.928
		5.28	2.955
	Mean = 2.9 ± 0.017		
Chinese	Allicin	5.33	1.822
		5.33	1.799
		5.38	1.813
	Mean = 1.811 ± 0.011		
NARC-G1	Allicin	5.22	4.823
		5.21	4.820
		5.25	4.821

Mean = 4.821 ± 0.01.

garlic is a sterile crop and grown vegetatively, it shows a prominent variation in phenotypic characteristics including the size of the bulb, weight of the cloves, plant height, and the number of cloves. Earlier studies have reported that bulb size and weight significantly affect the plant height which helps in fixing the essential compounds for photosynthesis (Panthee et al., 2006), consequently making plant height integral in increasing the plant's overall growth (Lencha and Buke, 2017). On the contrary, in our study there was exception to the general trend of positive correlation of bulb size with plant height and number of leaves. However, NARC-G1 showed unique attributes where no significant correlation between the plant height and number of leaves detected as NARC-G1 garlic bulb weighs almost three times more than other cultivars. The NARC G-1 plant showed higher bulb weight along with thicker stem resulting in the growth of the strong plant rather than theoretical expectations i.e., tall plant with more numbers of leaves. This trend was consistent across five repeats in same experimentation settings. Our study showed notable exception to previously reported findings by Ipek et al. (2005) that the genotype of the plant influences the number of cloves per bulb and weight of clove in a garlic bulb, where NARC-G1 comprised the least number of cloves per plant but the weight and width of the single cloves were double than all the other cultivars. Consequently, affecting the strength and growth of NARC-G1 garlic even if the mean of plant height and bulbs size is comparatively lower than Chinese garlic. In this study, we concluded exceptional findings related to NARC-G1 where the plant makes up lesser numbers of cloves however, the weight and size of a single clove are much greater than the other cultivars.

Essential elements of garlic reported to have integral role in regulating blood circulation, bone formation, blood-sugar control, and metabolism (Sher et al., 2012). The geographic variation highly affects the genetic attributes of garlic influencing the concentration of various elements in plant (Camargo et al., 2010). Since no elemental analysis study previously reported for NARC-G1, therefore, we performed a comparative elemental analysis of NARC-G1 with other cultivars to pinpoint the elemental differentiation. Garlic is known to have good amount of Fe, a vital nutrient essential

Table 2. Indels in *alliinase* gene of all the garlic cultivars.

Garlic Variety	Insertion		Deletion site	
	After Site	Sequence	Site	Sequence
Italian	a) 1048	a) TAAAATGA	a) 144-147	a) TAA
	b) 1773	b) ATGAATGA	b) 534-536	b) AA
	c) 1953	c) ATTAA		
	d) 1985	d) GCAACACAAGC		
Desi Gulabi	a) 376	a) ACGG	a) 428-429	a) AC
	b) 1766	b) TATGAATGACTGATGCA	b) 166-169	b) ATAC
	c) 1985	c) ATAGG		
Chinese	a) 350	a) GT	a) 45	a) A
	b) 491	b) GGGAC	b) 454-457	b) ATTA
	c) 1718	c) CATATGAATGACTGATC	c) 107-109	c) ATA
	d) 1890	d) ATG	d) 1052-1079	d) ATAACCCAGAAGGTCTGCTTCGCCATG
	e) 1932	e) GATG	e) 1088-1100	e) AGGGATGCAAAT
	f) 1972	f) CC		
NARC-G1	a) 920	a) TACT	a) 154-159	a) TTATTA
	b) 935	b) ATCTG	b) 406-414	b) GTTGATCTC
	c) 950	c) ATCTGTA	c) 425-435	c) TTCACACACAC
			d) 444-466	d) TATAATATCAATGTAGCTATAGC
			e) 1996	e) AC

for the cellular activities of all living organism while Si play integral role in building the bone mineral density and bone formation (Price et al., 2013; Ancuceanu et al., 2015). Our data showed essential elements: Fe and Si were only detected in NARC-G1 unlike previously reported data where Si and Fe are detected in all garlic type (Sajid et al., 2014; Petropoulos et al., 2018). These results suggest a further exploration of physiological mechanism involve in the synthesis of Fe and Si in various garlic species. Furthermore, Ca found in highest concentration only in NARC-G1. No traces of Zn and Mn were detected in NARC-G1 garlic as compared to the rest of the cultivars. Vanadium (V) known to be an essential element for treating diabetes is found in traces in NARC-G1 and Desi Gulabi, this trend of elements concentration is consistent with earlier studies (Khan et al., 2016; Polyakov et al., 2020). While discussing the significances of minerals in garlic exceptionally we found traces of Cd in all the garlic cultivar with the maximum amount in NARC-G1 garlic which could be dangerous and reported to be a carcinogenic (Kellen et al., 2007). One plausible explanation for the presence of Cd in garlic could be the contamination of soil and water with heavy metals. Cadmium is a pervasive heavy metal in nature accumulated due to industrial discharge, fossil fuel burning and sewage disposal (Singh et al., 2013). Garlic is reported to have a substantial amounts of heavy metals accumulation like cadmium and lead in monocultures and interplanting due to high underground biomass and extensive root system (Jiang et al., 2001). This suggests that the soil and water sources may have heavy metal contamination. Pakistan is known to have extensive water and soil contamination of heavy metals. Different agricultural practices including the irrigation of sewage water resulting in penetration of heavy metals in the food chain (Waseem et al., 2014; Nawab et al., 2015; Hussain et al., 2021). Because of current study's limitations, agronomic practices i.e., the soil health and water irrigation practices were not focused thus, the elemental analysis revealed the contamination of toxic heavy metal (Cd).

The quantity of bioactive compounds varies from cultivar to cultivar imparting the effect on active compounds content (Prati et al., 2014; Szychowski et al., 2018). To expand the comparison of NARC-G1 and other garlic cultivars, we performed HPLC based quantification to comparatively study the organo-sulfur compounds especially allicin. The HPLC results showed the highest concentration of allicin detected in NARC-G1. The obtained HPLC chromatogram depicted sharp peaks confirming that the NARC-G1 is two-fold richer in allicin concentration in comparison to all the selected garlic cultivars. According to British Pharmacopoeia (1998), the minimum allicin content to ensure pharmaceutical and economic viability of garlic powder products is 4.5 mg/g. Yeh and Liu (2001) and Yang et al. (2001) reported that allicin is an active biochemical compound with potent medicinal and therapeutic effects thus, the higher concentration of allicin in NARC-G1 (4.82 ± 0.001 mg/g) than all the other cultivars make it the best choice for therapeutic, pharmaceutical, and food consumption.

Alliinase enzyme is a core contender to initiate the metabolic pathway to produce allicin (Lancaster et al., 1988; Kim et al., 2009; Lanzotti, 2012). At the molecular level, the alliinase enzyme is encoded by the *alliinase* gene alternatively called alliin lyase. As study by Ovesná et al. (2015) detailed the nature of the gene and suggests the gene shows prominent level of nucleotide variation (indels) affecting the expression of the enzyme responsible to produce organosulfur compounds in garlic. In parallel to morpho-biochemical studies, we performed a detailed PCR based genotyping of the *alliinase* gene in NARC-G1 and all the cultivars to identify and compare the level of nucleotide variation in the *alliinase* gene. Our result coincides with the earlier studies showing the major nucleotide insertions and deletions in the *alliinase* gene among all the garlic varieties. In NARC-G1, maximum deletions of nucleotide sequences and insertion of three to seven nucleotides at various locations were found in the *alliinase* gene. These extensive nucleotide variations in NARC-G1 garlic could be the reason for imparting higher content of bioactive molecule allicin, making it a pharmaceutical and economic viable garlic variety. For future studies, we can expand the genetic analysis for the variation to whole-genome through transcriptome analysis for identification of nucleotide variation and polymorphism in NARC-G1 garlic.

5. Conclusion

In this novel study for comparative analysis of NARC-G1 with other garlic cultivars, we performed a detailed comparison to identify the phenotypic traits, chemical & analytical composition, and genetic variations. All the results suggest that NARC-G1 is a unique garlic cultivar with a large bulb size and desirably higher allicin content. Moreover, the elemental and analytical assessments suggest NARC-G1 garlic is a good nutritional garlic cultivar. Given all the results we conclude that NARC-G1 could be beneficial for industrial, nutritional, and pharmaceutical use. Additionally, due to NARC-G1 outstanding morphological traits, it can be the best choice for agriculture purposes.

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References

AMAGASE, H., 2006. Clarifying the real bioactive constituents of garlic. *The Journal of Nutrition*, vol. 136, no. 3, pp. 716S-725S. http://dx.doi.org/10.1093/jn/136.3.716S. PMid:16484550.

AMAGASE, H., PETESCH, B.L., MATSUURA, H., KASUGA, S. and ITAKURA, Y., 2001. Intake of garlic and its bioactive components. *The Journal of Nutrition*, vol. 131, no. 3, pp. 955S-962S. http://dx.doi.org/10.1093/jn/131.3.955S. PMid:11238796.

ANCUCEANU, R., DINU, M., HOVANEȚ, M.V., ANGHEL, A.I., POPESCU, C.V. and NEGREȘ, S., 2015. A survey of plant iron content: a semi-systematic review. *Nutrients*, vol. 7, no. 12, pp. 10320-10351. http://dx.doi.org/10.3390/nu7125535. PMid:26690470.

BOONPENG, S., SIRIPONGVUTIKORN, S., SAE-WONG, C. and SUTTHIRAK, P., 2014. The antioxidant and anti-cadmium toxicity properties of garlic extracts. *Food Science & Nutrition*, vol. 2, no. 6, pp. 792-801. http://dx.doi.org/10.1002/fsn3.164. PMid:25493198.

BRITISH PHARMACOPOEIA, 1998. *The pharmaceutical press*. London: Her Majesty's Stationary Office, vol. 1, 125 p.

CAMARGO, A., RESNIZKY, S., MARCHEVSKY, E. and LUCO, J., 2010. Use of the Argentinean garlic (*Allium sativum* L.) germplasm mineral profile for determining geographic origin. *Journal of Food Composition and Analysis*, vol. 23, no. 6, pp. 586-591. http://dx.doi.org/10.1016/j.jfca.2010.01.002.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS – FAO, 2015. *FAO statistical pocketbook 2015: world food and agriculture*. Rome: FAO.

FRY, F.H., OKARTER, N., BAYNTON-SMITH, C., KERSHAW, M.J., TALBOT, N.J. and JACOB, C., 2005. Use of a substrate/alliinase combination to generate antifungal activity in situ. *Journal of Agricultural and Food Chemistry*, vol. 53, no. 3, pp. 574-580. http://dx.doi.org/10.1021/jf048481j. PMid:15686404.

GONZÁLEZ, R.E., SOTO, V.C., SANCE, M.M., CAMARGO, A.B. and GALMARINI, C.R., 2009. Variability of solids, organosulfur compounds, pungency and health-enhancing traits in garlic (*Allium sativum* L.) cultivars belonging to different ecophysiological groups. *Journal of Agricultural and Food Chemistry*, vol. 57, no. 21, pp. 10282-10288. http://dx.doi.org/10.1021/jf9018189. PMid:19827749.

HUSSAIN, J., WEI, X., XUE-GANG, L., SHAH, S.R.U., ASLAM, M., AHMED, I., ABDULLAH, S., BABAR, A., JAKHAR, A.M. and AZAM, T., 2021. Garlic (*Allium sativum*) based interplanting alters the heavy metals absorption and bacterial diversity in neighboring plants. *Scientific Reports*, vol. 11, no. 1, p. 5833. http://dx.doi.org/10.1038/s41598-021-85269-4. PMid:33712650.

IPEK, M., IPEK, A., ALMQUIST, S. and SIMON, P., 2005. Demonstration of linkage and development of the first low-density genetic map of garlic, based on AFLP markers. *Theoretische und Angewandte Genetik*, vol. 110, no. 2, pp. 228-236. http://dx.doi.org/10.1007/s00122-004-1815-5. PMid:15565379.

IPEK, M., SAHIN, N., IPEK, A., CANSEV, A. and SIMON, P.W., 2015. Development and validation of new SSR markers from expressed regions in the garlic genome. *Scientia Agrícola*, vol. 72, no. 1, pp. 41-46. http://dx.doi.org/10.1590/0103-9016-2014-0138.

JIANG, W., LIU, D. and HOU, W., 2001. Hyperaccumulation of cadmium by roots, bulbs and shoots of garlic (*Allium sativum* L.). *Bioresource Technology*, vol. 76, no. 1, pp. 9-13. http://dx.doi.org/10.1016/S0960-8524(00)00086-9. PMid:11315815.

KELLEN, E., ZEEGERS, M.P., HOND, E.D. and BUNTINX, F., 2007. Blood cadmium may be associated with bladder carcinogenesis: the Belgian case–control study on bladder cancer. *Cancer*

Detection and Prevention, vol. 31, no. 1, pp. 77-82. http://dx.doi.org/10.1016/j.cdp.2006.12.001. PMid:17296271.

KHAN, H., HUSSAIN, M.M., JELLANI, G., TARIQ, S., NASEEB, T. and MAHMOOD, S., 2018. Evaluation of garlic genotypes for yield and yield components in Islamabad, Pakistan environment. *Nucleus*, vol. 55, no. 1, pp. 22-26.

KHAN, M.S., QUERSHI, N.A., JABEEN, F., ASGHAR, M.S. and SHAKEEL, M., 2016. Analysis of minerals profile, phenolic compounds and potential of garlic (*Allium sativum*) as antioxidant scavenging the free radicals. *International Journal of Biosciences*, vol. 8, no. 4, pp. 72-82. http://dx.doi.org/10.12692/ijb/8.4.72-82.

KHAN, N.H., 2021. Assessment of agronomic characters and yield in garlic genotypes under agro-climatic conditions of Mingora-Swat Pakistan. *Pure and Applied Biology*, vol. 10, no. 3, pp. 657-667. http://dx.doi.org/10.19045/bspab.2021.100068.

KIM, Y.S., BAEK, H., CHUNG, I., KWON, B. and JI, G., 2009. Garlic fermentation by lactic acid bacteria. *Food Science and Biotechnology*, vol. 18, no. 5, pp. 1279-1283.

KUMAR, M., SHARMA, V.R., KUMAR, V., SIROHI, U., CHAUDHARY, V., SHARMA, S., SARIPALLI, G., NARESH, R.K., YADAV, H.K. and SHARMA, S., 2019. Genetic diversity and population structure analysis of Indian garlic (*Allium sativum* L.) collection using SSR markers. *Physiology and Molecular Biology of Plants*, vol. 25, no. 2, pp. 377-386. http://dx.doi.org/10.1007/s12298-018-0628-y. PMid:30956421.

LANCASTER, J.E., DOMMISSE, E.M. and SHAW, M.L., 1988. Production of flavour precursors [S-alk(en)yl-l-cysteine sulphoxides] in photomixotrophic callus of garlic. *Phytochemistry*, vol. 27, no. 7, pp. 2123-2124. http://dx.doi.org/10.1016/0031-9422(88)80108-0.

LANZOTTI, V., 2012. Bioactive polar natural compounds from garlic and onions. *Phytochemistry Reviews*, vol. 11, no. 2-3, pp. 179-196. http://dx.doi.org/10.1007/s11101-012-9247-3.

LENCHA, B. and BUKE, T., 2017. Effects of clove size and plant density on the bulb yield and yield components of garlic (*Allium sativum* L.) in Sodo Zuria Woreda, Southern Wolaita Zone. *Journal of Natural Sciences Research*, vol. 7, no. 21, pp. 8-16.

LEONTIEV, R., HOHAUS, N., JACOB, C., GRUHLKE, M.C. and SLUSARENKO, A.J., 2018. A comparison of the antibacterial and antifungal activities of thiosulfinate analogues of allicin. *Scientific Reports*, vol. 8, no. 1, p. 6763. http://dx.doi.org/10.1038/s41598-018-25154-9. PMid:29712980.

LIU, D.S., WANG, S.L., LI, J.M., LIANG, E.S., YAN, M.Z. and GAO, W., 2017. Allicin improves carotid artery intima media thickness in coronary artery disease patients with hyperhomocysteinemia. *Experimental and Therapeutic Medicine*, vol. 14, no. 2, pp. 1722-1726. http://dx.doi.org/10.3892/etm.2017.4698. PMid:28810641.

MIKAIL, H.G., 2009. Effects of aqueous bulb extract of *Allium sativum* (garlic) on hematological and biochemical parameters in rabbits: experimental Trypanosoma brucei ssp. brucei infection. *Journal of Herbs, Spices & Medicinal Plants*, vol. 15, no. 3, pp. 265-271. http://dx.doi.org/10.1080/10496470903378979.

MIKAILI, P., MAADIRAD, S., MOLOUDIZARGARI, M., AGHAJANSHAKERI, S. and SARAHROODI, S., 2013. Therapeutic uses and pharmacological properties of garlic, shallot, and their biologically active compounds. *Iranian Journal of Basic Medical Sciences*., vol. 16, no. 10, pp. 1031-1048. PMid:24379960.

MOHAMMADI, B., KHODADADI, M., KARAMI, E. and SHAAF, S., 2014. Variation in agro-morphological characters in Iranian garlic landraces. *International Journal of Vegetable Science*, vol. 20, no. 3, pp. 202-215. http://dx.doi.org/10.1080/19315260.2013.788594.

NAWAB, J., KHAN, S., SHAH, M.T., QAMAR, Z., DIN, I., MAHMOOD, Q., GUL, N. and HUANG, Q., 2015. Contamination of soil, medicinal, and fodder plants with lead and cadmium present in mine-affected areas, northern Pakistan. *Environmental Monitoring and Assessment*, vol. 187, no. 9, p. 605. http://dx.doi.org/10.1007/s10661-015-4807-9. PMid:26324064.

NAZIR, I. and CHAUHAN, R.S., 2019. Qualitative phytochemical analysis of *Allium sativum* (Garlic) and *Curcuma longa* (Turmeric). *Journal of Entomology and Zoology Studies*, vol. 7, no. 1, pp. 545-547.

OVESNÁ, J., MITROVÁ, K. and KUČERA, L., 2015. Garlic (A. sativum L.) alliinase gene family polymorphism reflects bolting types and cysteine sulphoxides content. *BMC Genetics*, vol. 16, no. 1, p. 53. http://dx.doi.org/10.1186/s12863-015-0214-z. PMid:25997498.

PANTHEE, D.R., KC, R.B., REGMI, H.N., SUBEDI, P.P., BHATTARAI, S. and DHAKAL, J., 2006. Diversity analysis of garlic (*Allium sativum* L.) germplasms available in Nepal based on morphological characters. *Genetic Resources and Crop Evolution*, vol. 53, no. 1, pp. 205-212. http://dx.doi.org/10.1007/s10722-004-6690-z.

PERCIVAL, S.S., 2016. Aged garlic extract modifies human immunity. *The Journal of Nutrition*, vol. 146, no. 2, pp. 433S-436S. http://dx.doi.org/10.3945/jn.115.210427. PMid:26764332.

PETROPOULOS, S.A., FERNANDES, Â., NTATSI, G., PETROTOS, K., BARROS, L. and FERREIRA, I., 2018. Nutritional value, chemical characterization and bulb morphology of Greek garlic landraces. *Molecules*, vol. 23, no. 2, p. 319. http://dx.doi.org/10.3390/molecules23020319. PMid:29393882.

POLYAKOV, A., ALEKSEEVA, T. and MURAVIEVA, I., 2020. The elemental composition of garlic (Allium sativum L.) and its variability. *E3S Web of Conferences*, vol. 175, p. 01016. http://dx.doi.org/10.1051/e3sconf/202017501016.

PRATI, P., HENRIQUE, C.M., SOUZA, A.S., SILVA, V.S.N. and PACHECO, M.T.B., 2014. Evaluation of allicin stability in processed garlic of different cultivars. *Food Science and Technology*, vol. 34, no. 3, pp. 623-628. http://dx.doi.org/10.1590/1678-457x.6397.

PRICE, C.T., KOVAL, K.J. and LANGFORD, J.R., 2013. Silicon: a review of its potential role in the prevention and treatment of postmenopausal osteoporosis. *International Journal of Endocrinology*, vol. 2013, p. 316783. http://dx.doi.org/10.1155/2013/316783. PMid:23762049.

RABINKOV, A., MIRON, T., KONSTANTINOVSKI, L., WILCHEK, M., MIRELMAN, D. and WEINER, L., 1998. The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. *Biochimica et Biophysica Acta*, vol. 1379, no. 2, pp. 233-244. http://dx.doi.org/10.1016/S0304-4165(97)00104-9. PMid:9528659.

RIED, K. and FAKLER, P., 2014. Potential of garlic (*Allium sativum*) in lowering high blood pressure: mechanisms of action and clinical relevance. *Integrated Blood Pressure Control*, vol. 7, pp. 71-82. http://dx.doi.org/10.2147/IBPC.S51434. PMid:25525386.

ROGERS, S.O. and BENDICH, A.J., 1994. Extraction of total cellular DNA from plants, algae and fungi. In: S.B. GELVIN and R.A. SCHILPEROORT, eds. *Plant molecular biology manual*. Dordrecht: Springer. http://dx.doi.org/10.1007/978-94-011-0511-8_12.

SAJID, M., BUTT, M.S., SHEHZAD, A. and TANWEER, S., 2014. Chemical and mineral analysis of garlic: a golden herb. *Pakistan Journal of Food Sciences*, vol. 24, no. 2, pp. 108-110.

SAYADI, V., KARIMZADEH, G., MONFARED, S.R. and NAGHAVI, M.R., 2020. Identification and expression analysis of S-alk (en) yl-L-cysteine sulfoxide lyase isoform genes and determination of allicin contents in Allium species. *PLoS One*, vol. 15, no. 2, p. e0228747. http://dx.doi.org/10.1371/journal.pone.0228747. PMid:32092058.

SCHÄFER, G. and KASCHULA, C.H., 2014. The immunomodulation and anti-inflammatory effects of garlic organosulfur compounds in cancer chemoprevention. *Anti-cancer Agents in Medicinal Chemistry*, vol. 14, no. 2, pp. 233-240. http://dx.doi.org/10.2174/18715206113136660370. PMid:24237225.

SECKINER, I., BAYRAK, O., CAN, M., MUNGAN, A.G. and MUNGAN, N.A., 2014. Garlic supplemented diet attenuates gentamicin nephrotoxicity in rats. *International Braz J Urol*, vol. 40, no. 4, pp. 562-567. http://dx.doi.org/10.1590/S1677-5538.IBJU.2014.04.17. PMid:25251961.

SHER, A., FAKHAR-UL-MAHMOOD, M., SHAH, S.N.H., BUKHSH, S. and MURTAZA, G., 2012. Effect of garlic extract on blood glucose level and lipid profile in normal and alloxan diabetic rabbits. *Advances in Clinical and Experimental Medicine*, vol. 21, no. 6, pp. 705-711. PMid:23457127.

SINGH, A., KUMAR, C.S. and AGARWAL, A., 2013. Effect of lead and cadmium on aquatic plant *Hydrilla verticillata. Journal of Environmental Biology*, vol. 34, no. 6, pp. 1027-1031. PMid:24555332.

SOOD, D.R., CHHOKAR, V. and SINGH, J., 2000. Studies on growth, pungency, and flavour characteristics of five varieties of garlic, (*Allium sativum* L.) bulbs during development. *Vegetable Science*, vol. 27, no. 2, pp. 180-184.

SZYCHOWSKI, K.A., RYBCZYŃSKA-TKACZYK, K., GAWEŁ-BĘBEN, K., ŚWIECA, M., KARAŚ, M., JAKUBCZYK, A., MATYSIAK, M., BINDUGA, U.E. and GMINSKI, J., 2018. Characterization of active compounds of different garlic (*Allium sativum* L.) cultivars. *Polish Journal of Food and Nutrition Sciences*, vol. 68, no. 1, pp. 73-81. http://dx.doi.org/10.1515/pjfns-2017-0005.

WASEEM, A., ARSHAD, J., IQBAL, F., SAJJAD, A., MEHMOOD, Z. and MURTAZA, G., 2014. Pollution status of Pakistan: a retrospective review on heavy metal contamination of water, soil, and vegetables. *BioMed Research International*, vol. 2014, p. 813206. http://dx.doi.org/10.1155/2014/813206. PMid:25276818.

YANG, C.S., CHHABRA, S.K., HONG, J.-Y. and SMITH, T.J., 2001. Mechanisms of inhibition of chemical toxicity and carcinogenesis by diallyl sulfide (DAS) and related compounds from garlic. *The Journal of Nutrition*, vol. 131, no. 3, pp. 1041S-1045S. http://dx.doi.org/10.1093/jn/131.3.1041S. PMid:11238812.

YEH, Y.-Y. and LIU, L., 2001. Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies. *The Journal of Nutrition*, vol. 131, no. 3, pp. 989S-993S. http://dx.doi.org/10.1093/jn/131.3.989S. PMid:11238803.

YOO, M., LEE, S., LEE, S., SEOG, H. and SHIN, D., 2010. Validation of high-performance liquid chromatography methods for determination of bioactive sulfur compounds in garlic bulbs. *Food Science and Biotechnology*, vol. 19, no. 6, pp. 1619-1626. http://dx.doi.org/10.1007/s10068-010-0229-1.

Supplementary Material

Supplementary material accompanies this paper.

Appendix S1: Set of primers for amplification of overlapping region of alliinase gene.
Appendix S2: Agronomic traits of four garlic cultivars.
Appendix S3: Elemental data of essential and non-essential elements through ICP-OES in four garlic cultivars: Italian garlic, Desi Gulabi garlic, Chinese garlic and NARC-G1.

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