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# Exploring the impact of 1-deoxynojirimycin on alpha-galactosidase activity and chickpea seed germination through *in vitro* experiments and molecular docking analysis

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**ABSTRACT:** Seed germination is a tightly regulated physiological process. Hydrolytic enzymes provide energy that brings physiological, biochemical, and physical changes to the seed during germination. Alpha-galactosidases break down alpha 1-6 linked galactosyl groups in glycoproteins, glycolipids, and oligosaccharides into simple compounds to provide energy during seed germination. Chickpea seed germination characteristics showed that inhibition of alpha-galactosidase using 1-deoxynojirimycin (DNJ), a derivative of deoxygalactonojirimycin, delays germination. To gain insights into the mechanism of inhibition, we modeled the enzyme's structure and performed *in-silico* docking of the inhibitor and natural substrates (raffinose and stachyose) to the enzyme. We also validated our model using recombinant chickpea alpha-galactosidase. Our docking studies showed that both the inhibitor and the substrates bind to the same active site pocket in the enzyme but to different amino acid residues, stachyose being a preferred substrate, and DNJ competitively inhibits alpha-galactosidase with a Ki and IC50 of 44.5 µM and 89.13 µM respectively. Delayed germination is a desirable agronomic practice that can be explored for better crop establishment and to prevent pre-harvest sprouting in crops.

**Index terms:** 1-deoxynojirimycin, alpha-galactosidase, germination, inhibition kinetics.

**RESUMO:** A germinação das sementes é um processo fisiológico rigorosamente regulado. As enzimas hidrolíticas fornecem energia que traz mudanças fisiológicas, bioquímicas e físicas à semente durante a germinação. As alfa-galactosidases decompõem os grupos galactosil ligados a alfa 1-6 em glicoproteínas, glicolipídeos e oligossacarídeos em compostos simples para fornecer energia durante a germinação das sementes. As características de germinação das sementes de grão de bico mostraram que a inibição da alfa-galactosidase com 1-desoxinojirimicina (DNJ), um derivado da desoxigalactonojirimicina, atrasa a germinação. Para obter informações sobre o mecanismo de inibição, modelamos a estrutura da enzima e realizamos o acoplamento *in-silico* do inibidor e dos substratos naturais (rafinose e estaquiose) à enzima. Também validamos nosso modelo usando alfa-galactosidase recombinante de grão de bico. Nossos estudos de *docking* mostraram que tanto o inibidor quanto os substratos se ligam ao mesmo arcabouço de sítio ativo na enzima, mas a diferentes resíduos de aminoácidos, sendo a estaquiose um substrato preferido, e o DNJ inibe competitivamente a alfa-galactosidase com um Ki e IC50 de 44,5 µM e 89,13 µM, respectivamente. O atraso na germinação é uma prática agronômica desejável que pode ser explorada para um melhor estabelecimento das culturas e para evitar a germinação pré-colheita nas culturas.

**Termos para indexação:** 1-desoxinojirimicina, alfa-galactosidase, germinação, cinética de inibição.

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## INTRODUCTION

Germination is an integrated process involving the physiology of the seed, enzymatic regulation of the seed metabolism, and tight regulation of gene expression (Xue et al., 2020). Seed germination in chickpea consists of three phases: phase 1, the hydration of the seed where the seed imbibes water; phase 2, the hydrated seed kick starts the enzymatic machinery, mostly hydrolases to break down complex reserves to simple compounds that serves various purposes during germination, and phase 3, the commitment to germination where the radicle breaches the seed barrier to complete germination (Bewley et al., 2013; Pereira et al., 2022).

Enzymes, especially hydrolytic enzymes, are crucial in cell wall modification during seed germination (Bewley et al., 2013). Further, the energy produced by enzymatic hydrolysis of sugar (Han and Yang, 2015), starch (Joshi, 2018), and seed coat glucans (Simmons, 1994) are used to fuel seed germination. In addition, cysteine proteases unfold and breakdown reserve proteins (Tully et al., 1978; Dunaevsky et al., 1989), whereas lipases hydrolyze ester carboxylate bonds to release fatty acids and organic alcohols (Barros et al., 2010).

It is noteworthy that Raffinose Family of Oligosaccharides (RFOs), though present in all parts of the plant, accumulate at higher levels in the legume seed. The RFOs act as an energy reserve for germination, promote seed longevity, and protect cells against abiotic stresses (Elango et al., 2022). The RFOs are hydrolyzed by alpha-galactosidases that cleave the terminal galactosyl residue to yield galactose and sucrose (Rackis, 1975). The sucrose is cleaved to glucose and fructose by invertase to meet the energy needs of seed germination. The alpha-galactoside levels decrease as the seed germinates, with an associated increase in alpha-galactosidase enzyme activity, indicating their crucial role in seed germination (Arunraj et al., 2020).

Inhibiting the seed hydrolytic enzymes can prevent or postpone germination to overcome unfavorable agronomic conditions, post-harvest seed preservation, enhance long-term storage of germplasm, and prevent pre-harvest sprouting of seeds in crop plants (Chenyin et al., 2023). Thus, enzyme inhibitors are critical tools used not only to understand enzyme's role in seed germination but enhance the understanding of germination physiology to explore seed germination inhibition as an agronomic tool for crop improvement (Evenari, 1949). In this study, we investigated the effect of deoxynojirimycin, a derivative of deoxygalactonojirimycin, on chickpea seed germination. We performed *in-silico* simulations of enzyme-inhibitor interactions and validated the mechanism of inhibition through *in vitro* experiments.

## MATERIAL AND METHODS

### *Chickpea seed germination with inhibitors DNJ*

Chickpea seeds (Chirag) procured from Ankur seeds, Nagpur, India was used in this study. The seeds were washed in running tap water and surface sterilized using 1% sodium hypochlorite for 3 minutes. The seeds were washed three times in copious amounts of sterile water each for 5 minutes. The surface sterilized seeds were imbibed overnight (16 hours) in an aqueous solution of DNJ (100 µM). The imbibed seeds were germinated on a moist tissue soaked with DNJ. The control seeds were imbibed in sterile water and sprouted on a wet tissue soaked in sterile water. Germination characteristics including germination percentage (GI), mean germination time (MGT), mean germination rate (MGR), synchronization index (Z), coefficient of variation of germination time ($CV_t$), and coefficient of velocity of germination (CVG), were computed every 24 hours up to 96 hours after imbibition (Al-Ansari and Ksiksi, 2016). The rooting index was calculated using the given formula.

$$\text{Rooting index} = (\text{length on the } 1^{st} \text{ day}/1) + (\text{length on the } 2^{nd} \text{ day}/2) + \ldots\ldots (\text{length on the } n^{th} \text{ day}/n)$$

Seed vigor, a critical measure that indicates the seed's potential for germination, was calculated as the product of germination percentage and the seedling length (Abdul-Baki and Anderson, 1973).

$$GV = \text{Germination \%} \times \text{seedling length}$$

### *Homology modeling of Cicer alpha-galactosidase*

Since X-ray diffraction and Nuclear Magnetic Resonance (NMR) spectroscopic structures of alpha-galactosidase were unavailable in the Protein Data Bank (PDB), the amino acid sequence of chickpea alpha-galactosidase was subjected to Blastp, and the retrieved reference sequence was used to build a homology model (Table 1).

Homology modeling was performed using both template-based (SWISS-MODELLER and I-TASSER) (Zhang et al., 2017; Zheng et al., 2021) and non-template-based approaches (IntFOLD (McGuffin et al., 2017; McGuffin et al., 2019) and PHYRE 2 (Kelley et al., 2015). SWISS-MODELLER and I-TASSER predicted five models each, while IntFOLD and PHYRE 2 generated five and one model, respectively.

To predict enzyme active site and inhibitor binding sites, I-TASSER and GRaSP were used (Santana et al., 2020). The structural model of alpha-galactosidase was used to dock both the substrates (raffinose and stachyose) and the inhibitor deoxynojirimycin using Autodock Vina 4 (Trott et al., 2010; Eberhardt et al., 2021). The DNJ structure was downloaded from the PubChem database with compound CID number 29435. The grid box dimension values used for docking were center_x=-32.562; center_y=17.832; center_z=-41.007, size_x=70, size_y=50, size_z=70. The binding scores and binding residues for the ligands were recorded.

### *Alpha-galactosidase and enzyme assay*

Recombinant alpha-galactosidase from *Cicer arietinum* was used in this study. Alpha-galactosidase-specific activity was estimated using the artificial substrate pNPG; the assay reaction consisted of 90 µL of the enzyme with 10 µL of pNPG (5mM) in a total of 200 µL with McIlvaine buffer pH 5.4. The assay mixture was incubated in the dark at 37 °C for 30 minutes. The reaction was terminated by adding 1mL of 3M sodium carbonate, and the product n-nitrophenol was measured at 405 nm. The enzyme-specific activity was calculated using the following formula:

$$\text{Gal units} = (\text{OD405 X Reaction volume})/(\text{Extinction coefficient X Reaction time X Enzyme volume})*(10^5)$$

### *Enzyme inhibition assay*

Deoxynojirimycin (1-Deoxynojirimycin hydrochloride from Sigma Aldrich (CAS Number-73285-50-4)) is a known inhibitor of alpha-galactosidase (Asano et al., 2000). Before the enzyme assay, the enzyme was pre-incubated with the inhibitor for 30 minutes (Kuzmic, 2020). The DNJ concentration used for the enzyme inhibition study was 100 µM. The assay was performed with substrate concentrations (0mM, 1mM, 2mM, 3mM, and 4mM) and the type of inhibition characterized. Ki, the inhibition constant, and the IC50 value were calculated using the Lineweaver-Burk plot (LB plot) (Brandt et al., 1987; https://www.sciencegateway.org/protocols/cellbio/drug/hcic50.html).

Table 1. Top blastp hits for the amino acid sequence of Alpha galactosidase from Cicer areitinum LOC101502309. Search filters were set to protein data bank proteins. (Source link: https://blast.ncbi.nlm.nih.gov/Blast.cgi).

| Scientific Name | Max score | Total score | Query cover | E-Value | Per-Ident | Acc len | Accession |
|---|---|---|---|---|---|---|---|
| *Nicotiana benthamiana* | 584 | 584 | 85% | 0 | 76.52% | 363 | 6F4C_B |
| *Oryza sativa* | 531 | 531 | 85% | 0 | 69.15% | 362 | 1UAS_A |
| *Umbelopsis vinacea* | 291 | 291 | 84% | 8.00E-95 | 40.57% | 397 | 3A5V_A |
| *Bacteroides fragilis* NCTC 9343 | 273 | 273 | 83% | 1.00E-86 | 40.68% | 477 | 4NZJ_A |
| *Gallus gallus* | 266 | 266 | 85% | 4.00E-85 | 38.66% | 405 | 1KTB_A |
| *Bacteroides fragilis* NCTC 9343 | 265 | 265 | 83% | 1.00E-83 | 40.32% | 473 | 4OGZ_A |
| *Homo sapiens* | 254 | 254 | 72% | 1.00E-80 | 43.60% | 398 | 1R46_A |
| *Homo sapiens* | 253 | 253 | 72% | 4.00E-80 | 43.60% | 404 | 3LX9_A |
| *Homo sapiens* | 252 | 252 | 72% | 2.00E-79 | 43.29% | 404 | 3HG3_A |
| *Saccharomyces cerevisiae* | 250 | 250 | 81% | 8.00E-78 | 39.18% | 479 | 3LRK_A |

*Statistical analysis*

The experiments were done in biological and technical replications. The germination data was analyzed on GraphPad Prism 8.0 using a paired 't' test at the significance level $p<0.05$ (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA).

## RESULTS AND DISCUSSION

Seed germination is a tightly regulated complex physiological process estimated by various indices over time to gain insight into the potential germination characteristics during seed germination (Khan et al., 2023). The alpha-galactosidase inhibitor, DNJ reduced the germination rate in chickpeas. The impact of DNJ on the percent germination, mean germination rate (MGR), frequency of germination, coefficient of variation of germination time ($CV_t$), and total length of the seedling were insignificant 96 hours after imbibition (Figure 1). However, the inhibitory effect of DNJ on mean germination time (MGT), the average length of time required for the maximum germination, coefficient of the velocity of germination (CVG), and seed vigor were significant at $p<0.05$ (Figure 1). The results suggest that the impact of DNJ on germination was reversible, as evident from the temporal variation in the germination indices. These findings corroborate the delay in soybean seed germination treated with 1-deoxygalactonojirimycin (Dierking and Bilyeu, 2009). Enzyme inhibitor-induced delayed germination is agronomically favourable to overcome adverse conditions, weed management, and germplasm preservation (Chenyin et al., 2023).

To understand enzyme-inhibitor interaction, molecular docking and inhibition kinetics were performed. Sixteen models generated by SWISS-MODELLER, I-TASSER, IntFOLD, and PHYRE 2 were validated sequentially using the ERAT score and Ramachandran plot (Table 2) to select four homology-based models. The four models' superimposition

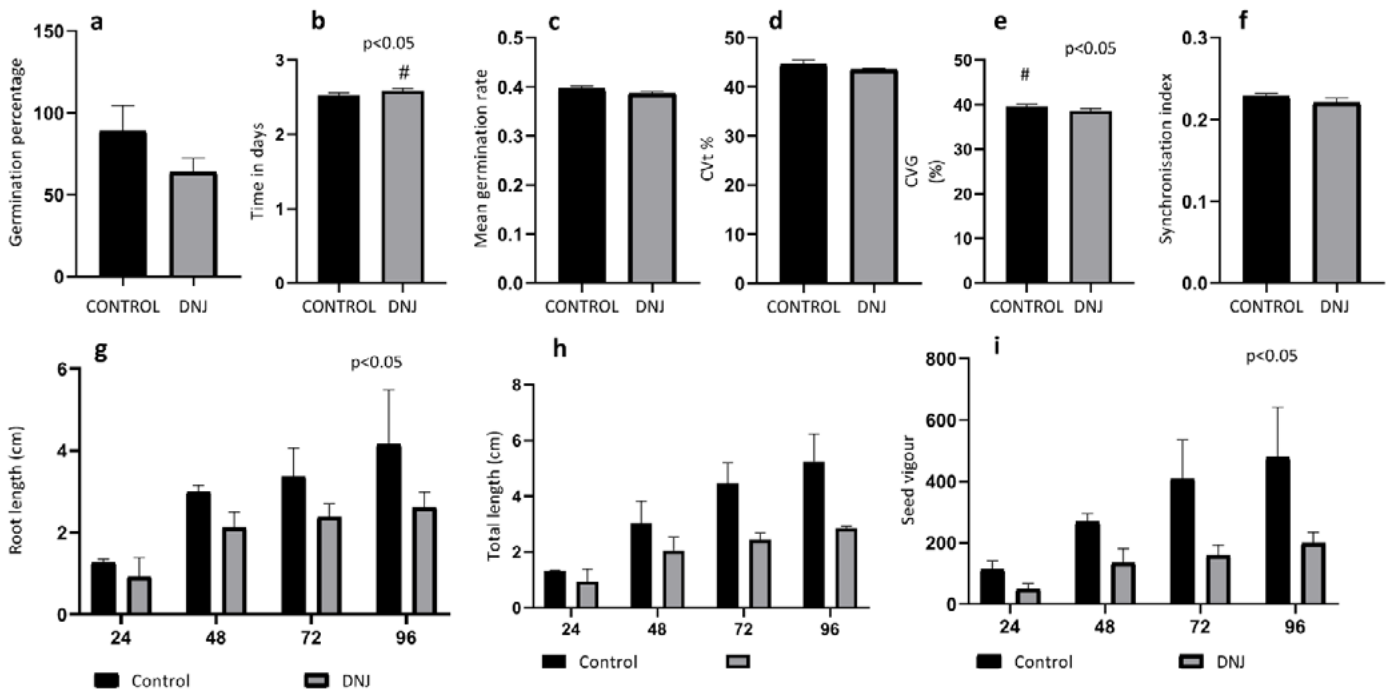


Figure 1. The germination characteristics of the effect of inhibitor on seed germination were computed as germination percentage (a), mean germination time (b), mean germination rate(c), coefficient of variation of germination time (d), coefficient of velocity of germination (e), synchronization index (f), root length (g), total length of the seedling (h), and seed vigour (i). The experiment was performed in biological duplicates with n=10. The variation in the germination indices MGT, CVG, root length, and seed vigour were statistically significant at $p < 0.05$.

(Figure 2a) shows that the most reliable homology-based alpha-galactosidase model was that SWISS-MODELER predicted (Figure 2b). I-TASSER and GRASP predicted the enzyme's binding pocket and active site (Table 3). Docking with Auto dock Vina 4 showed that the enzyme bind to the natural substrates (raffinose and stachyose) and inhibitor deoxynojirimycin within the binding pocket (Figure 3a). Auto docking results display the H-bonds and hydrophobic interactions between the enzyme and substrate/inhibitor at the active site (Figures 3b, c, d and Table 4). The binding affinity of the substrates (raffinose and stachyose) and the inhibitor are -7.92, -3.61, and -6.89, respectively (Table 4). The docking simulations revealed that the natural substrates and the inhibitor bind to the same pocket in the predicted active site but to different amino acid residues, perhaps due to structural and steric differences. The occurrence of the conserved 'CEW' domain (Cys 222, Trp 224) in the binding pocket ratifies our docking model; the CEW domain is crucial for enzyme-inhibitor interaction (Motabar et al., 2010).

The LB plot (Figure 4) defines Km as substrate concentration at which the reaction rate is half maximum. The LB plot exhibited variance in the slope but with equal Y-intercept, implying competition between substrate and inhibitor for enzyme binding (Waldrop, 2009). The DNJ competitively binds to alpha-galactosidase, inhibiting the hydrolysis of oligosaccharide reserve that source energy, causing a considerable delay in seed germination. The Ki, inhibitor constant and IC50, inhibitor concentration that inhibits half the enzyme activity calculated from the Y-intercept of LB plot was 44.5 µM and 89.13 µM respectively (Liclican et al., 2020; Butt et al., 2023). The Ki indicates the stability of the enzyme-inhibitor complex; a Ki <100 µM suggests a stronger binding affinity between the enzyme and inhibitor (Zheng and Polli, 2010).

Table 2. Screening of the most reliable homology model using SAVES and Ramachandran plot using Procheck. (Source link: https://saves.mbi.ucla.edu/)

| Model | ERAT | Ramachandran plot | | | |
|---|---|---|---|---|---|
| | | Core | Allowed | Generously allowed | Disallowed |
| Phyre 2 | 77.55 | 85.3 | 14.1 | 0.3 | 0.3 |
| SWISS 1 | 95.7265 | 87.5 | 11.3 | 0.6 | 0.6 |
| SWISS 2 | 96.01 | 86.2 | 13.2 | 0.3 | 0.3 |
| SWISS 3 | 89.3 | 86.5 | 11.6 | 1 | 1 |
| SWISS 4 | 83.23 | 86.2 | 11.3 | 1.9 | 0.6 |
| SWISS 5 | 93.29 | 86.5 | 11.9 | 0.6 | 1 |
| I-TASSER 1 | 80.09 | 70.3 | 20.5 | 7 | 2.2 |
| I-TASSER 2 | 77.66 | 65.1 | 26.5 | 6.2 | 2.2 |
| I-TASSER 3 | 80.58 | 70 | 21.4 | 6.8 | 1.9 |
| I-TASSER 4 | 66.26 | 60.8 | 27 | 7 | 5.1 |
| I-TASSER 5 | 85.64 | 66.2 | 27 | 5.7 | 1.1 |
| IntFold 1 | 95 | 81.9 | 15.4 | 2.2 | 0.5 |
| IntFold 2 | 92.7 | 75.4 | 16.5 | 5.9 | 2.2 |
| IntFold 3 | 93.66 | 78.4 | 17.6 | 1.9 | 2.2 |
| IntFold 4 | 92.05 | 74.6 | 14.1 | 6.5 | 4.9 |
| IntFold 5 | 92.5 | 78.6 | 15.4 | 2.4 | 3.5 |

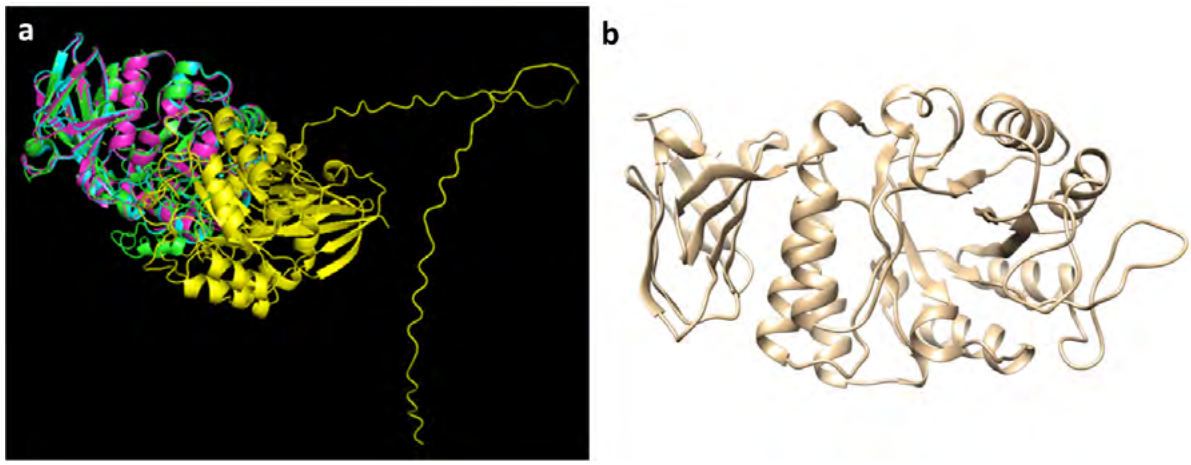


Figure 2. Superimposed image of the generated homology based models developed using Pink- I-TASSER, Cyan- Swiss model, Green – Pyre, and Yellow- Intfold (a). Alpha-galactosidase structural model of computed by SWISS MODELLER (b).

Table 3. Binding pocket of the model was visualized using and active sites were predicted using I-TASSER (Zheng et al., 2021) and GRasp (Santana et al., 2020).

| Amino acid residues in Binding pocket | | | Amino acid residues in Active site | |
|---|---|---|---|---|
| | | | I-TASSER | GRaSP |
| L-65 | Y-186 | T-242 | W-76 | W-76 |
| W-76 | L-187 | T-243 | D-111 | D-111 |
| Y-107 | D-190 | G-244 | D-112 | D-112 |
| I-108 | F-218 | D-245 | Y-153 | Y-153 |
| N-109 | S-220 | K-249 | C-161 | D-155 |
| L-110 | L-221 | S-252 | K-188 | C-161 |
| D-111 | C-222 | M-253 | D-190 | K-188 |
| D-112 | E-223 | I-254 | C-222 | D-190 |
| G-151 | W-224 | D-255 | W-224 | W-224 |
| C-161 | S-239 | D-256 | R-241 | R-241 |
| S-162 | R-241 | Q-259 | D-245 | D-245 |

Further, since enzyme inhibition is concentration dependent, an increase in the natural substrate (RFOs) concentration with progress in germination out-competes the inhibitor, thus reversing the initial inhibition, justifying only a delay in germination, but not total inhibition. In addition, germination is a complex physiological process tightly regulated at the physiological and molecular levels. Integrative regulation of several factors, including hormone balance, seed-micro-environment interaction, and hydrolytic enzymes, play a critical role in seed germination. Alpha-galactosidases contribute to this complex network by hydrolyzing trisaccharide and tetrasaccharide reserves of galactose (RFOs) into simple sugars to energetically favor germination (Arunraj et al., 2020).

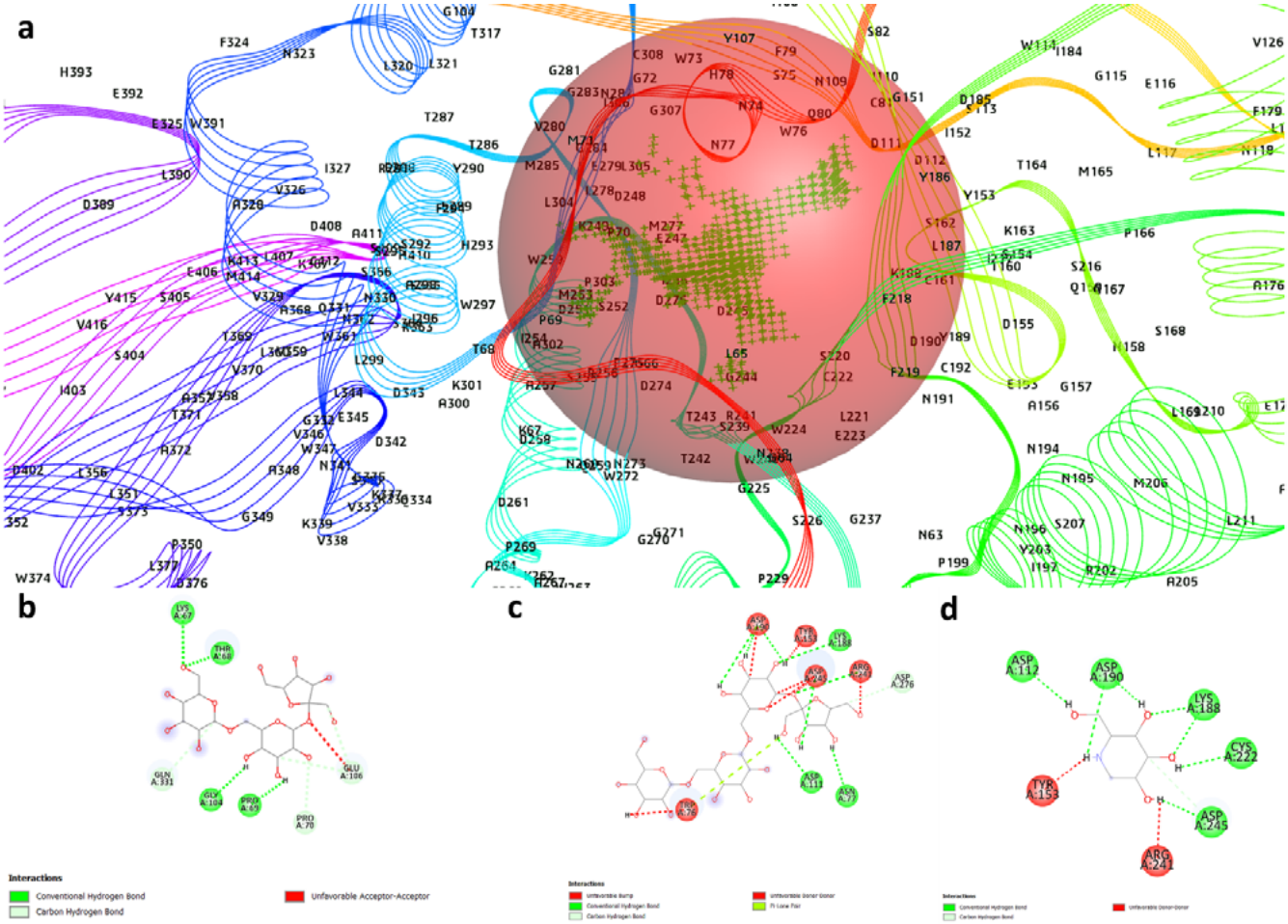


Figure 3. The binding pocket in alpha-galactosidase predicted by *in silico* homology modeling (a) is shown. Molecular docking using Autodock vina 4 shows the binding of substrates (raffinose (b) and stachyose(c)), and inhibitor DNJ (d) to the active site of alpha-galactosidase. The various color code indicates the type of interaction between ligand and receptor.

Table 4. Docking score analysis with hydrogen and hydrophobic interactions by Autodock vina (Eberhardt et al., 2021).

| S.No | Compound | Binding affinity | H-Bond | Non-Hydrogen bonds |
|---|---|---|---|---|
| 1 | DNJ | -6.89 | Asp 112, Asp 190, Lys 188, Cys 222, Asp 245 | Thr 160, Ser 162, Trp 76, Asp 111,Trp 224 |
| 2 | Raffinose | -7.92 | Lys 67, Thr 68, Pro 69, Gly 104 | Pro 70, Glu 106, Gln 331 |
| 3 | Stachyose | -3.61 | Asn 77, Asp111, Lys 188 | Trp 76 , Tyr 153, Asp 190, Arg 241, Asp245 |

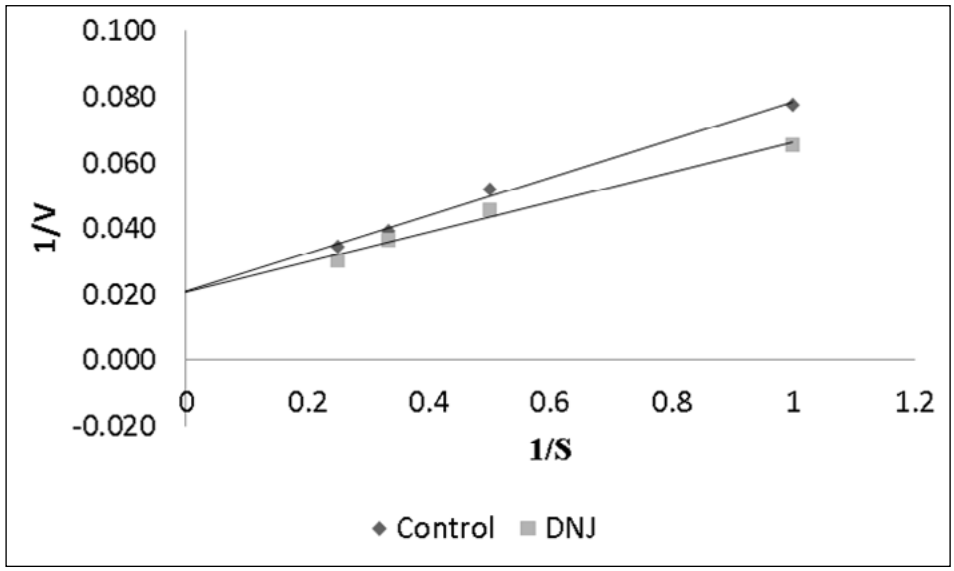


Figure 4. Lineweaver-Burk plot, exhibits the type of inhibition between alpha-galactosidase and DNJ. The enzyme's rate of reaction against various substrate concentrations indicates that the type of inhibition is competitive inhibition.

## CONCLUSIONS

The findings suggest that DNJ competitively inhibits alpha-galactosidase to delay seed germination. Delayed germination is a potential tool to leverage over unfavorable agronomic and post-harvest storage conditions. Further studies are required to explore the implications of delayed germination in crop establishment and performance.

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