ANTAGONISTIC EFFECTS OF SELECTED SOIL-BORNE BACTERIA ON RALSTONIA SOLANACEARUM AND TOMATOES DISEASE, GROWTH AND YIELD

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Abstract
The study investigated antagonistic effects of selected soil-borne bacteria on Ralstonia solanacearum, tomatoes disease, growth and yield assessment. Three (3) selected soil-borne bacteria: Bacillus subtilis, Bacillus cereus and Pseudomonas fluorescens and one (1) control group with no bacterium were allotted equally to Root Dip (RD) and seed coating (SC) inoculation randomly in 4 x 2 factorial arrangements completely randomized design. The RD (selected soil-borne bacteria/4-week-old seedlings) and SC (2.5 x 10^7 cfu/mL of each selected soil-borne bacteria/25g seed) antagonistic effects on Ralstonia solanacearum were assessed in a greenhouse experiment. Also, Disease Severity (DS, 1: no symptom to 5: dead plants), Disease Incidence (DI, %), Plant Height (PH, cm), Shoot Weight (SW, g), Root Weight (RW, g) and Fruit Yield (FY, t/ha) were measured in greenhouse. Data were analyzed using ANOVA at α<0.05. Results show that the three selected soil-borne bacteria inhibited the growth of Ralstonia solanacearum in vitro (1.5 - 4.0 cm). Bacillus subtilis (ST) had least DS (1.5±0.02), DI (36.2), highest PH (36.8±0.04), SW (22.0±0.05), RW (1.6±0.03) and FY (8.0±0.02), while Pseudomonas fluorescens (RD) had highest DS (2.6±0.04), DI (74.5), least PH (21.3±0.03), SW (13.2±0.05), RW (1.1±0.02) and FY (3.2±0.03) in greenhouse. The SC method of inoculation controlled Ralstonia solanacearum better and produced higher tomato growth and yield than RD method. Thus, seed coated with Bacillus subtilis (2.5 x 10^7 cfu/mL/25g seed) increased tomato growth and yields, and reduced incidence and severity of the pathogen. Bacillus subtilis, is therefore recommended as a good bio-control agent of R. solanacearum; and for increase tomato growth and yields.

Keywords: R. solanacearum, Disease, Growth, Yield, Tomato-seed dressing, Root dip

Introduction
Tomato (Solanum lycopersicon L.) is a vegetable crop cultivated worldwide and consumed in fresh or processed form (Li Niu, Dyck, Wang & Zou, 2016). About 40 million tonnes of tomatoes was processed worldwide to produce tomato juice, paste, puree, ketchup, canned tomatoes and many other products (WPTC, 2015). Tomato possesses a high content of vitamins, foliates, carotenoids and phenolic
compounds (Savatovic, Cetkovic, Canadovic-Brunet & Dilas, 2010), and essential amino acids, fatty acids and minerals (Elbadrawy & Sello, 2016; Violeta, Tatiana, Maviana, Raluca & Alexandru, 2018).

According to FAO (2016), China as the largest producer, with an output of 59.5 million tonnes representing 33% of 182.3 million tonnes world production in 2017. Egypt is the largest producer in Africa followed by Nigeria with 4.1 million tonnes. In 2018, global tomato market generated revenue of $190.4 billion (Globe News Wire, 2019). In spite of the huge amount of revenue generated by tomato, its production is seriously threatened by bacterial diseases (Nion & Toyota, 2015). Cultivation of tomato in Nigeria is severely affected by bacterial wilt caused by the soil-borne pathogen, _Ralstonia solanacearum_. (Adebayo & Ekpo, 2005). Survey of major tomato producing areas in South western Nigeria showed epidemic of bacterial wilt with high incidences of 60-80% in fields. High yield loss of over 70% was reported in major agro-ecological zones in Nigeria (Adebayo, 2011).

Different measures have been put in trial to achieve its control; either by inhibiting pathogen growth within the rhizosphere or by inducing host plant resistance (Ding, Shen, Zhang & Chen, 2013; Jogaiha, Abdelrahman, Tran & Shin-ichi, 2013), but limited success has been achieved due to high surviving capacity of _Ralstonia solanacearum_ in complex environments (King, Davis, Liu & Levi, 2008). Various bactericides have been used but with their limitations (Li, 2015). Broad host range and high variability of strains of _Ralstonia solanacearum_ make it difficult to make use of resistant varieties (Ji, Momol, Meister, Norman & Jones 2007). Chemical bactericides are associated with toxic residues and environmental pollution (Butt, Jackson & Magan, 2011).

Biological control is a non hazardous alternative for integrated pest management. It is the suppression of populations of plant pathogens by living organisms (Heimpel & Mills, 2017). Antagonists are form of microorganisms that inhibit the growth of other microbes. The populations of antagonists are established on plant surfaces to compete with pathogens for sites and nutrients (Priou, Marquez & Guatra, 2006). Ability of biocontrol bacteria to efficiently colonize the rhizosphere is a key factor in the improvement of plant health and suppression of plant pathogens (Zhang, 2011; Chowdhury, 2013).

Biological agents are applied to crops for biological control of plant pathogens where they act via a range of mode of actions. Some interact with plants by inducing resistance or priming plants without any direct interaction with the targeted pathogens. Antagonists acting through hyper-parasitism and antibiosis are directly interfering with the pathogens through synthesis of enzymes or other interfering metabolites. In most cases, antimicrobial metabolites are produced by antagonists directly on the spot where the targeted organism is
harmful. Applied antagonists modulate growth conditions and make it less favourable for pathogen development.

Various studies have indicated the use of antagonistic bacteria to reduce incidence of bacterial wilt disease of tomato. Toyota and Kimura (2001) reported the suppressive effect of an avirulent mutant on the bacterium, *Ralstonia solanacearum*. Some other naturally occurring antagonistic rhizobacteria that have been used include *Pseudomonas fluorescens* (Guo, 2004), *Bacillus* species (Wydra, Diogo, Pannon & Samaru, 2005) and *Pythium oligandrum*. Various actinomycete and Bacillus bacteria such as *Bacillus mesentericus, B. megaterium, B. subtilis* and *B. mycoides* have been reported by Doan and Nguyen (2006) as active biological control agents.

Although the potential to suppress the pathogenic organisms through biological means has been revealed, but sufficient information has not been generated so far to fully support the development of biological control measures. Therefore, there is a need to investigate the antagonistic effect of selected soil-borne bacteria on *Ralstonia solanacearum* and tomatoes disease, growth and yield.

**Materials and Methods**

The experimental design was completely randomized design in 4 x 2 factorial arrangements comprising three (3) selected soil-borne bacteria: *Bacillus subtilis, Bacillus cereus* and *Pseudomonas fluorescens* and one (1) control group with no bacterium were allotted equally to Root Dip (RD) and Seed Coating (SC) inoculation methods. The experiment was carried out in Pathology Laboratory of Crop protection and Environmental Biology Department, University of Ibadan, Ibadan. The pot experiment was conducted in the greenhouse of the same department.

Materials such as glass rods, forceps, inoculation wire loop, pins and needles were heated until red-hot before use. Glassware were washed in Teepol detergent and rinsed with tap water. Washed Petri dishes were put in canisters, pipettes were wrapped in aluminum foil, and all glassware were sterilized in a hot air oven at 160° C for one hour. Scalpsels were sterilized by dipping in 70 percent alcohol and passing through flame in an inoculating chamber during usage. Soil samples 0 -15 cm layers were randomly collected using soil auger from Department of Crop Protection and Environmental Biology experimental plots. The soil was weighed and 5 kg was transferred into pots of 20 cm surface diameter, 15 cm base diameter and 25 cm depth in the greenhouse for planting.
Top-soil was loaded into a trough, moistened and steamed for twelve hours at 120°C. The soil was weighed and 5 kg portion was transferred 20 cm into pots of surface diameter, 15 cm base diameter and 25 cm depth in the greenhouse for planting. The pathogenic organism, *Ralstonia solanacearum* was isolated from wilted tomato plants showing typical symptoms of bacterial wilt. The plant roots were thoroughly washed in tap water and surface sterilized by dipping in 70% ethanol for 1 minute and allowed to be air dried. The plant roots were chopped into 1 cm pieces and put into sterile water in sterile capped bottle (Denny, 2006).

The chopped roots were maintained in the water for 30 minutes to allow the bacteria diffuse. After 30 minutes in water, two loopfuls of the water suspension were streaked onto triphenyl tetrazolium chloride (TTC) medium containing peptone (10 g), dextrose (10 g), cassamino acid, (1 g), agar (18 g) and distilled water and aqueous solution of TTC (1 liter). The medium was incubated at 30°C for 48 hrs. After 48 hrs of incubation, purification of *Ralstonia. solanacearum* colony was done by temporarily maintaining the isolates in distilled water. Identification of *Ralstonia. solanacearum* colonies was made when typical colonies showed a characteristic red center and whitish periphery on TTC medium (Kelman, 1954).

The selected soil-borne bacteria were *Bacillus cereus*, *B. subtilis*, *Pseudomonas fluorescens*. *Ralstonia. solanacearum* was paired with each antagonist on nutrient agar plates (Hartman, Hong, Hanudin & Hayward, 1993). A loopful of *B. cereus* (2.5 x 10⁷ cfu/mL), *B. subtilis* 2.5x10⁷ cfu/ml) and *P. fluorescens* (2.5 x 10⁷ cfu/mL), then incubated for 48 hrs. After 48 hrs of incubation, a loopful of *R. solanacearum* (2.5 x 10⁷ Cf/mL) was used to streak across the middle of each plate. Control plates had no antagonists. All the treatments were incubated at 28 °C for 72 hrs.

Tomato seeds were uniformly coated with 2.5 x 10⁷ cfu/mL each of *B. subtilis*, *B. cereus* and *P. fluorescens*, while those for control group were coated with sterile distilled water. The seeds were air-dried for 24 hrs and planted in a pot of 20 cm surface diameter, 15 cm base diameter and 25 cm depth containing 5 kg of steam sterilized before being infested by mixing 2.5 x 10⁷ cfu/ml suspension of *Ralstonia solanacearum* in each pot. Three seeds were planted in each of 4 replicated pots per antagonist.

For Root dipping method, 15 seeds of tomato, Ibadan local were planted in trays containing steam sterilized soil for 4 wks in the nursery. Roots of seedlings were washed using sterile distilled water before dipping in 1 liter suspension each of *B. subtilis*, *B. cereus* and *P. fluorescens* at 2.5 x 10⁷ cfu/mL.
for 30 minutes. Seedlings were transplanted into pot containing 5 kg of steam sterilized soil. At transplanting, the seedlings were inoculated by pouring 2.5 x 10⁷ cfu/mL suspension of *Ralstonia solanacearum* round the base of the seedlings (Hartman *et al*., 1993; De cal, Pasca, Larena & Melgarejo, 1995).

The seedlings were watered every other day till harvest. The seeds treated by root dipping treatment and seed coating were planted on the same day. The seeds treated by root dipping treatment were transplanted after 4 wks. Data on plant height, fresh shoot weight, root weight, disease incidence, severity and population of *Ralstonia solanacearum* in the rhizosphere were collected at 35 days after transplanting for root dipping treatments and 63 days after sowing for seed coating treatment. Three randomly selected wilted plants per pot were used to estimate the population of *Ralstonia solanacearum* in the rhizosphere of tomato plants. Each root sample (0.1g) was placed in 10ml of sterile distilled water, after shaking for 5 minutes, ten-fold dilutions were prepared from the stock and 0.ml of dilution 10³ and 10⁴ were spread each on TTCA. Plates were incubated at 30°C for 48hrs and typical fluidal colonies of *Ralstonia.solanacearum* were counted.

Wilted plants were recorded for each treatment twice weekly. The proportion of wilted plants based on the number of plants using the percentage incidence as shown in the formula below:

\[
\text{% Incidence} = \frac{n}{N} \times 100
\]

Where n is the number of plants showing wilt symptoms with at least one leaf wilted, and N is the total number of sampled plants used in the experiment. Plant height was measured with a meter rule in cm, and shoot and root weight was measured with weighing balance in g.

Plants were assessed for wilt severity 30 days after inoculation. A scale of 1-5 after the methods of He, Ly, Sequeira & Kelman (1983) was used as follows:

- no symptom;
- one leaf wilted;
- two or three leaves wilted;
- four or more leaves wilted; and
- whole plant dead.
Data were subjected to analysis of variance (ANOVA) at $\alpha 0.05$ and means separation was achieved using Duncan’s Multiple Range.

**Results**

*In vitro* experiment of soil-borne bacteria antagonism effect on *Ralstonia solanacearum*

The result of in vitro experiment of soil-borne bacteria (*Bacillus cereus, Bacillus subtilis* and *Pseudomonas fluorescens*) antagonism effect on *Ralstonia solanacearum* showed growth inhibition as indicated in Table 1.0.

Table 1.0: *In vitro* experiment of soil-borne bacteria antagonism effect on *Ralstonia solanacearum*

<table>
<thead>
<tr>
<th>Soil-borne bacteria</th>
<th>Zone of inhibition (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus,</em></td>
<td>2.5</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>4.0</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>1.5</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The antagonists formed zone of inhibition restricted growth of *Ralstonia solanacearum*. The antagonism was shown to be highest for *Bacillus subtilis* (4.0 cm), while *Bacillus cereus* and *Pseudomonas fluorescens* had inhibitions of 2.5 cm and 1.5 cm respectively. The inhibitions of the three soil-borne bacteria were higher compared with the control group (no antagonist).

**Effect of soil-borne bacteria antagonism and inoculation method on wilt incidence, severity and root population of *Ralstonia solanacearum* in greenhouse experiment**

The effect of using soil-borne bacteria antagonism had significant effect ($P<0.05$) on the wilt incidence and severity of bacterial wilt of tomato (Table 2.0).
Table 2.0: Effect of soil-borne bacteria antagonism and inoculation method on wilt incidence, severity and root population of *Ralstonia solanacearum*

<table>
<thead>
<tr>
<th>Inoculation Method</th>
<th>Soil-borne bacteria</th>
<th>Wilt incidence (%)</th>
<th>Severity (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed coating</td>
<td><em>Bacillus subtilis</em></td>
<td>36.2e</td>
<td>1.48c</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em></td>
<td>52.5d</td>
<td>2.20b</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>56.0d</td>
<td>2.30b</td>
</tr>
<tr>
<td></td>
<td>No antagonist</td>
<td>85.00a</td>
<td>4.40a</td>
</tr>
<tr>
<td>Root dipping</td>
<td><em>Bacillus subtilis</em></td>
<td>63.5c</td>
<td>2.10b</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em></td>
<td>72.4b</td>
<td>2.40b</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>74.5b</td>
<td>2.60b</td>
</tr>
<tr>
<td></td>
<td>No Antagonist</td>
<td>85.20a</td>
<td>4.33a</td>
</tr>
</tbody>
</table>

Means followed by different letters along column are significantly different.

The wilt incidence (36.5-56.0%) and severity (1.8-2.3) in plants treated with antagonists as seed coating were significantly (P<0.05) lower than wilt incidence (63.5-74.5%) and severity (2.1-2.6) of plants treated with antagonists through root dipping. Wilt incidence (36.5-74.5%) and severity (1.8-2.6) in plants treated...
with antagonists were significantly (P<0.05) lower than wilt incidence (85.0 and 85.2%) and severity (4.3 and 4.4) in control group. Lowest wilt incidence (36.5%) and severity (1.4) were obtained in *Bacillus subtilis* treated plants as seed coating, while highest wilt incidence (74.5%) and severity (2.6) were recorded in *Pseudomonas fluorescens* treated using root dipping. The effect of inoculation method on soil population of *Ralstonia solanacaaru* showed that all the antagonists applied using the two methods of inoculations had no significant effect on the soil population of *R. solanacearum*. The range of 6.9 - 7.3 cfu/g population was observed in antagonist treated plants which were not significantly different from the soil population of control group (7.3 and 7.5 cfu/ml). The least soil population of *Ralstonia solanacearum* was obtained in *Bacillus. subtilis* treated plants as seed coating while highest soil population was obtained in control group (7.5 cfu/g).

**Effect of soil-borne bacteria and inoculation method on the growth and yield of tomato in greenhouse**

The effect of soil-borne bacteria antagonism on the plant height of tomato seedlings from 5th to 8th week after transplanting (WAT) showed no significant differences in the plant height between the treated plants and control up till 6th WAT (Table 3.0).

Table 3.0: Effect of soil-borne bacteria antagonism and inoculation methods on tomato growth and yield

<table>
<thead>
<tr>
<th>Inoculation Method</th>
<th>Soil-borne bacteria</th>
<th>Plant height WAT (cm)</th>
<th>Shoot weight (g)</th>
<th>Root weight (g)</th>
<th>Fruit Yield (t/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Seed coating</td>
<td><em>Bacillus subtilis</em></td>
<td>12.0b</td>
<td>15.2b</td>
<td>25.9a</td>
<td>36.8a</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em></td>
<td>11.4b</td>
<td>13.5c</td>
<td>22.5b</td>
<td>32.5b</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>11.3b</td>
<td>13.2c</td>
<td>25.5b</td>
<td>31.8c</td>
</tr>
<tr>
<td>No Antagonist</td>
<td>15.0a</td>
<td>16.4b</td>
<td>16.7d</td>
<td>17.5e</td>
<td>8.5d</td>
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<tr>
<td>Root dipping</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>15.8a</td>
<td>19.4a</td>
<td>20.9c</td>
<td>22.3d</td>
<td>14.2c</td>
</tr>
<tr>
<td><em>Bacillus Cereus</em></td>
<td>15.8a</td>
<td>18.5a</td>
<td>19.5c</td>
<td>22.1d</td>
<td>13.8c</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>15.7a</td>
<td>18.0a</td>
<td>19.5c</td>
<td>21.3d</td>
<td>13.2c</td>
</tr>
</tbody>
</table>

Means followed by different letters along column are significantly different

At 8th WAT, plant heights (21.3 cm – 36.8 cm) of soil-borne bacteria treated plants were significantly higher than control group (7.5 and 7.2). Plant heights were significantly (p<0.05) taller in soil-borne bacteria applied as seed dressing (31.8 -36.8cm) than the control group (17.5 and 17.2cm). When the two methods of inoculation were compared, highest plant height (36.8cm) was observed in each other plant heights applied through root dipping (21.3-22.3). Plant heights (21.3 36.8 cm) in all treated *subtilis* applied as seed dressing, while least plant height (21.3 cm) was in *P. fluorescens* applied through root dipping. Taller plants were recorded in soil-borne bacteria applied as seed dressing. Comparison of soil-borne bacteria showed highest plant heights in *B. subtilis* applied as seed dressing (36.8 cm) and through root dipping (22.3 cm), while *P. fluorescens* applied through root dipping (21.3 cm) and as seed treatment( 31.8 cm) had least plant heights. Shoot weights (13.2 -22.0 g) and root weights (1.1 -1.6 g) of soil-borne bacteria treated plants were significantly (P<0.05) higher than shoot weight (8.5 and 9.0 g) and root weight (0.2 g) in control group in the two methods of inoculation (Table 3.0). Shoot weights (19.5 -22.0g) of soil-borne bacteria treated plant as seed coating were significantly higher than the shoot weights (13.2 – 14.2g) of soil-borne bacteria treated plant as root dipping. Root weights (1.1 – 1.6g) in soil-borne bacteria treated plants as seed coating were
significantly higher (except in \textit{Pseudomonas fluorescens}) than soil-borne bacteria treated plant as root dipping (1.1g).

Fruit yield (3.2-8.0 t/ha) of tomato plants treated with soil-borne bacteria were significantly higher than control (0.4 t/ha) (Table 3.0). Soil-borne bacteria treated plants as seed coating had significantly higher fruit yield (4.8-8.0 t/ha) than plants treated with soil-borne bacteria through dipping (3.2-4.4 t/ha). Highest fruit yield was obtained in \textit{Bacillus subtilis} treated plants as seed dressing, while least fruit yield (8.0 t/ha) was recorded in \textit{Pseudomonas fluorescens} treated plants as root dipping (3.2 t/ha).

**Discussion**

\textit{In vitro} studies on the screening of \textit{Bacillus subtilis}, \textit{B. cereus} and \textit{Pseudomonas fluorescens} as potential antagonists showed their effectiveness by inhibiting growth of \textit{Ralstonia solanacearum}. This is supported by the study of Abdalla, Al-Mihanna, Al-Rokibah and Ibrahim (1998) that \textit{Bacillus subtilis} has a potential bio-control for tomato bacterial wilt. \textit{Bacillus subtilis} was observed as the best antagonist in screening, this is in consonance with report of Wang, et al. (2017) that \textit{Bacillus} strains produce antibiotic and a better bio-control agent because of its additive synergistic effect.

The greenhouse studies showed that \textit{Bacillus subtilis}, \textit{Bacillus cereus} and \textit{Pseudomonas fluorescens} were good bio-control agents on \textit{R. solanacearum}. Their efficacy was shown by increasing plant height, shoot weight, root weight and fruit yield, with significant reduction in tomato wilt incidence and severity of \textit{R. solanacearum}. Wydra and Samaru (2005) have reported that \textit{Ralstonia} wilt reduction and yield increase were associated with bio-control agents using \textit{Bacillus} and \textit{Pseudomonas} species. They reported significantly higher biomass and plant height over the control of \textit{R. solanacearum} with the use of \textit{Bacillus} and \textit{Pseudomonas} species. The three antagonists suppress \textit{R. solanacearum} of tomato by inducing systemic resistance or antibacterial activity (Nion & Toyota, 2015). This suggest that some plant defense enzyme activities were induced by the three antagonists according to Anand (2007). Soil-borne bacteria may have induced systemic physiological change in plant metabolism and enhance crop’s anabolic processes as stated by Abdalla \textit{et al.} (2006) and later improved tomato yield as reported by Abdalla & Ezzat (2007).

Effectiveness of these soil-borne bacteria on \textit{R. solanacearum} was observed in the two methods of inoculation. Seed dressing method of inoculation impacted \textit{R. solanacearum} higher tomato growth and yield than dipping method. However, significant reduction in plant height recorded in tomato plant in
seed dressing inoculation from 1st to 5th week after transplanting (WAT) could be as a result of delay that occurred in their seed germination from the coating of the seeds when compared with the root dipping. Soil-borne bacteria were unable to reduce soil population of *R. solanacearum*. It is suggested that the antagonists may have hindered migration of *R. solanacearum* into the vascular bundles (as shown by the reduction in wilt incidence and severity) but not significantly affected by population according to Jurgen *et al.* (2016).

**Conclusion**

*Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fluorescens* in vitro inhibited the growth of *R. solanacearum*, reduced incidence of bacterial wilt and increased tomato growth in greenhouse experiment. The soil-borne bacteria applied as seed treatment performed better than root dipping but with a delay in germination of their seeds for 5 week after planting.

**Recommendations**

Based on the findings of this study it was recommend that application of *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fluorescens* at a rate of $2.5 \times 10^7$ cfu/mL as seed coating method effectively reduce bacterial wilt and increase tomato growth and yield.

**References**


Globe News Wire, (2019). The world tomato market analysis, forecast, size and insights


