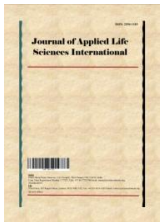

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Comparative Analysis of Different Nutrient Media for Growth of *Agrobacterium tumefaciens* under Small Volume Cultures

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Authors' contributions

This work was carried out in collaboration among all authors. Author AI designed the study, wrote the protocol and conducted the experiments. Authors AI and ND performed the statistical analysis, wrote the first draft of the manuscript and managed the literature searches. Author TK managed the analyses of the study and edited the first draft. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the effects of different selective complex growth media: Yeast Extract Peptone (YEP), Yeast Extract Mannitol (YEM), and Luria Bertani (LB) on growth and multiplication of three different strains of *Agrobacterium tumefaciens.*

Place and Duration of Study: Molecular Biology Laboratory, Genetics and Tree Improvement Division, Arid Forest Research Institute, Jodhpur, Rajasthan, India, between July and December 2020.

Methodology: Three strains of *Agrobacterium* (EHA105, GV3101 and LBA4404) were inoculated to grow on three different complex nutrient media (YEP, YEM, LB) supplemented with respective antibiotics for a period of 30 hours. Optical Density (O.D) at 600 nm was measured at every 3-hour interval to analyze differential growth pattern.

Results: Though all the three nutrient media supported the growth of bacteria, YEP medium supported the fast growth of all strains of bacteria, LB medium was a little less efficient but comparable to YEP media. However, YEM medium proved the least supportive for bacterial growth among the three media types.

Conclusion: *Agrobacterium* is used for genetic transformation of plant species and crop improvement. knowledge of the growth pattern of bacteria is necessary for the effective infection of

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plant material. We have analyzed that three strains of *Agrobacterium* on three different complex media, among the three media YEP media was supporting fast growth. This study can help the researchers to gain knowledge of the growth pattern of *Agrobacterium* to be used in the genetic transformation of plant species accordingly.

Keywords: Agrobacterium; nutrient medium; growth curve; Optical Density (O.D).

1. INTRODUCTION

As bacteria are easy to grow in the lab, their growth has been studied extensively. It has been determined that in a closed-system bacterium grows in a predictable manner, resulting in a growth curve composed of four distinct phases of growth: the lag phase, the exponential or log phase, the stationary phase, and the death or decline phase. Provided with the right conditions (food, correct temperature, etc.) microbes can grow very quickly. It's important to have knowledge of their growth requirements, so we can predict or control their growth under a particular set of conditions.

Agrobacterium tumefaciens also known as *Rhizobium radiobacter* is an aerobic, gram-negative, motile, rod-shaped soil bacterium of about 1.5-3.0 x 0.6-1.0 µm in size *Agrobacterium tumefaciens* is the causative agent of crown gall disease in plants [1] through the insertion of its T-DNA into the host genome and this ability has enabled it to be used as a tool in producing transgenic plants. The use of *Agrobacterium* not only shortens the conventional plant breeding process but also allows for entirely different genes to be engineered into crops. The story of *Agrobacterium* goes even further than this, making it one of the most interesting and significant bacteria for detailed study. Here we studied the growth of three strains of *Agrobacterium* over three different complex and selective nutrient broths, that comprises of a variety of carbon sources and other minerals for optimal growth. YEM [2], YEP [3], and LB [4,5] broths that can be prepared in the lab and available commercially, were studied for supporting the growth of three strains of *Agrobacterium*: GV3101, EHA105 (virulent strain developed from supervirulent wild type strain A281) [6,7] and LBA4404 [8] for a period of 30 hours in a controlled set of the conditions in small cultures (10 ml).

Yeast Extract Mannitol (YEM) broth is a complex medium widely used for the cultivation of *Agrobacterium* species and other soil microorganisms like *Rhizobium* species to make it suitable for the production of legume inoculants [9]. YEM broth contains yeast extract as a source for nitrogen and growth factors and mannitol, a fermentable sugar alcohol as a carbon source for *Agrobacterium*. It also maintains the oxidation-reduction potential of the medium in the range favourable for microorganisms and serves as a hydrogen donor in the respiratory process [10]. One limitation of YEM broth could be the poor growth of some strains due to variations in nutritional requirements [11].

Yeast Extract Peptone (YEP) is another complex medium widely used for the cultivation of *Agrobacterium* and other soil microorganisms. It has yeast extract, peptone, and sodium chloride as its main constituent. Yeast extract and peptone provide nitrogenous compounds, vitamin-B complex, and other growth nutrients for the growth of *Agrobacterium*. Sodium chloride maintains the osmotic balance of the medium. It is based on the formula described by Song *et a*l. [12]. Its efficiency can be limited as some strains may show poor growth due to nutritional variations [13].

LB medium, also known as Luria-Bertani medium or Lysogeny Broth, initially formulated by Giuseppe Bertani in 1951 for studying lysogeny in *Escherichia coli* [4,5], is a widely used rich medium to grow bacteria of many species as it is easy to prepare and provides a broad base of nutrients. It is comprised of tryptone, yeast extract, NaCl. The use of LB broth becomes inappropriate in physiological studies wherein reproducibility is required [14]. Therefore, this study aimed to assess the effects of different selective complex growth media: Yeast Extract Peptone (YEP), Yeast Extract Mannitol (YEM), and Luria Bertani (LB) on growth and multiplication of three different strains of *Agrobacterium tumefaciens*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Three strains of *Agrobacterium tumefaciens* were used in this study, EHA105 [6,7], GV3101,

LBA4404 [8] derived from a less virulent strain Ach5. All these three strains are being used in the genetic transformation of monocots and dicot plant species.

2.2 Nutrient Media

Three selective complex media broths were used to test their efficiency in supporting the growth of *Agrobacterium* under similar conditions with respective antibiotics specific for each strain. Ready to use Yeast Extract Mannitol (YEM), Yeast Extract Peptone (YEP), Luria Bertani Broth (LB), were obtained from Himedia labs, India, the composition is shown in Table 1. 25 grams of mixture for LB, 11.80 grams of mixture for YEM, and 25 grams of mixture for YEP media is suspended in 1000 ml of distilled water then the medium was buffered to pH 7.0 and sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. Rifampicin (10 mg/l) and other respective antibiotics i.e streptomycin (25 mg/l) specific for LBA4404, chloramphenicol (50 mg/l) for EHA105, gentamycin (10 mg/l) for GV3101 were added in the medium after sterilization to allow growth of only desired bacterial strain [15].

2.3 Place and Duration of Study

This study was conducted at Molecular Biology Laboratory in Genetics and Tree Improvement Division of Arid Forest Research Institute, Jodhpur, Rajasthan, India, between July and December 2020.

2.4 Culture Conditions

A single colony from the fresh plate of each *Agrobacterium* strains: LBA4404, EHA105, GV3101 was inoculated in 10 ml of all nutrient broth containing respective antibiotic (mentioned above) in falcon tubes and allowed to grow under the same condition at 28°C with continuous shaking at 180 RPM over orbital shaker (Orbitek, India). Three replicates of each strain over each medium were set up for experimentation.

2.5 Growth Curve

Optical Density (O.D.) is a measure of the turbidity of the bacterial growth and was measured as absorbance at 600 nm, at every 3-hour interval for each of the nine cultures and their three replicates and mean of all three replicate readings ± standard error (SE) were plotted against time (hour) to analyses the growth of bacterial strains over different nutrient broths.

2.6 Microscopic Examination

Microscopic examination of every culture was done by Gram staining [16] using crystal violet and counter-staining by safranin and visualized under a light microscope at a magnification of 40x objective lens.

2.7 Statistical Analysis

The growth analysis experiments were performed with three replications per treatment. Each experiment was repeated at least twice, and the reported data are the means of one experiment. All data presented as mean ± standard error (SE). Data were analyzed with IBM SPSS software version 22. Differences were determined by one-way analysis of variance (ANOVA), and significant ($P \leqslant 0.01$) differences among mean values were estimated using Duncan's multiple range test (DMRT).

Table 1. Composition and characteristic of nutrient media for 1-liter volume

YEM broth	YEP broth	LB broth
Yeast extract (1 g)	Peptone (10 g)	Tryptone (10 g)
Mannitol (10 g)	Yeast extract (10 g)	Yeast extract (5 g)
Dipotassium hydrogen phosphate (0.50 g)	Sodium chloride (5 gm)	Sodium chloride (10 g)
Magnesium sulphate (0.20 g)		
Sodium chloride (0.10 g)		
pH 7.0 ± 0.2	pH 7.0 ± 0.2	pH 7.5 ± 0.2
Light yellow colour	Light Brown colour	Yellow colour

3. RESULTS

A total of 9 growth curves at different time points were shown. The shapes of the growth curves were slightly different depending on the nutrient medium used and the strain tested (Fig. 1A, 1B, and 1C). However, they were reproducible among the replicates and the strains tested. Since the results of the two experiments were similar, the data of the first experiment were used for analysis. Since *Agrobacterium* is slowly multiplying, the growth of three strains was analyzed on various nutrient broths. All three strains have been inoculated in three complex media, i.e., YEP. YEM and LB to aid the growth of bacterial strains and to find growth supporting efficiencies of these nutrient broths. The growth curve and statistical analysis showed that all three bacterial strains (EHA105, LBA4404, GV3101) were able to grow at a different pace on three nutrient media: YEP, YEM, LB. Yeast extract Peptone (YEP) showed to promote the best growth of all three strains among the three-nutrient media. In terms of growth, LB was slightly lower but comparable to the YEP Broth. However, the growth and multiplication rates of bacteria were the least among the three in the YEM media. A similar growth pattern was observed for all bacterial strains on three growth media by plotting the O.D versus time graph.

Similar results were also obtained when data was statistically analyzed (Table 2) for $O.D._{max}$ at the 24^{th}-hour time point for all the media and strains, showing that YEP media was more supportive in the growth of bacteria in comparison to LB and YEM. Microscopic observation of bacterial cultures was also performed by Gram staining, which showed that cultures were pure and free from any contamination showing counter-stained rod-shaped bacteria (Fig. 2(b)).

4. DISCUSSION

Agrobacterium is slow-growing bacteria and being a natural plant genetic engineer, it is extensively modified by the researcher for faster and specific addition of desirable character in crops and plant species [17]. Microorganisms differ in their nutrition requirement and an optimal nutrient medium should be able to provide enough or adequate growth for the microorganisms. The relativity of this definition is evident since all the media tested, supports the growth of *Agrobacterium* strains (EHA105, GV3101, LB4404) to various degrees. Moreover, an adequate medium is a minimal requirement that has to be fulfilled for a medium to be considered as a suitable growth medium. Normally, most *Agrobacterium* strains will grow on a wide range of complex and defined media. Here, three complex-selective media (YEP, YEM, LB) were used, which are widely used for bacterial growth, we conducted this study to test their relative efficacy in supporting the growth of *Agrobacterium*-an important tool for plant genetic modification. It was shown that among the three mediums, YEP was performing better in terms of bacterial multiplication than LB which was comparable, and YEM, which was least performing among the three. YEP broth was performing better as it contains peptone in addition to yeast extract and sodium chloride, which provides nitrogen, amino acids, and minerals to the growing bacteria, which possibly act as a better source for growing bacteria in comparison to the tryptone in LB and mannitol in YEM. YEP media was also reported to increase the efficiency of *Agrobacterium* medium transient transformation of tropical maize genotypes when used in co-cultivation media [18].

The growth curve obtained in our experiments resembles normal bacterial growth curves like *Escherichia coli* [19] consisting of lag, exponential or log phase, decline, and stationary phase and It was reported that $O.D_{600}$ =1 corresponds to 5 x 10^8 cells/ml [20] which is also similar to *E.coli* (8x 10^8 cells/ml).

As *Agrobacterium* is slow multiplying, we have cultured the bacterial strains in a small amount of nutrient medium (10 ml) so the stationary or decline phase could be achieved faster and data was recorded for 30 hours. It was observed in other experiments (result not shown) that the time to reach the stationary phase gets extended accordingly if a higher volume of media is used and a single bacterial colony is inoculated in it, that is why small primary cultures are set-up before stepping up to the higher amount of cultures. With this observation, we have conducted the study in small culture batches to analyze the performance of nutrient mediums on the growth of *Agrobacterium*. This study helps to understand the *Agrobacterium* growth patterns, which is important if one wants to use it for genetic manipulation of plants as *Agrobacterium*-mediated transformation is widely been used for research in plant molecular biology and for genetic improvement of crops exploiting its tremendous ability to transfer foreign DNA to plants.

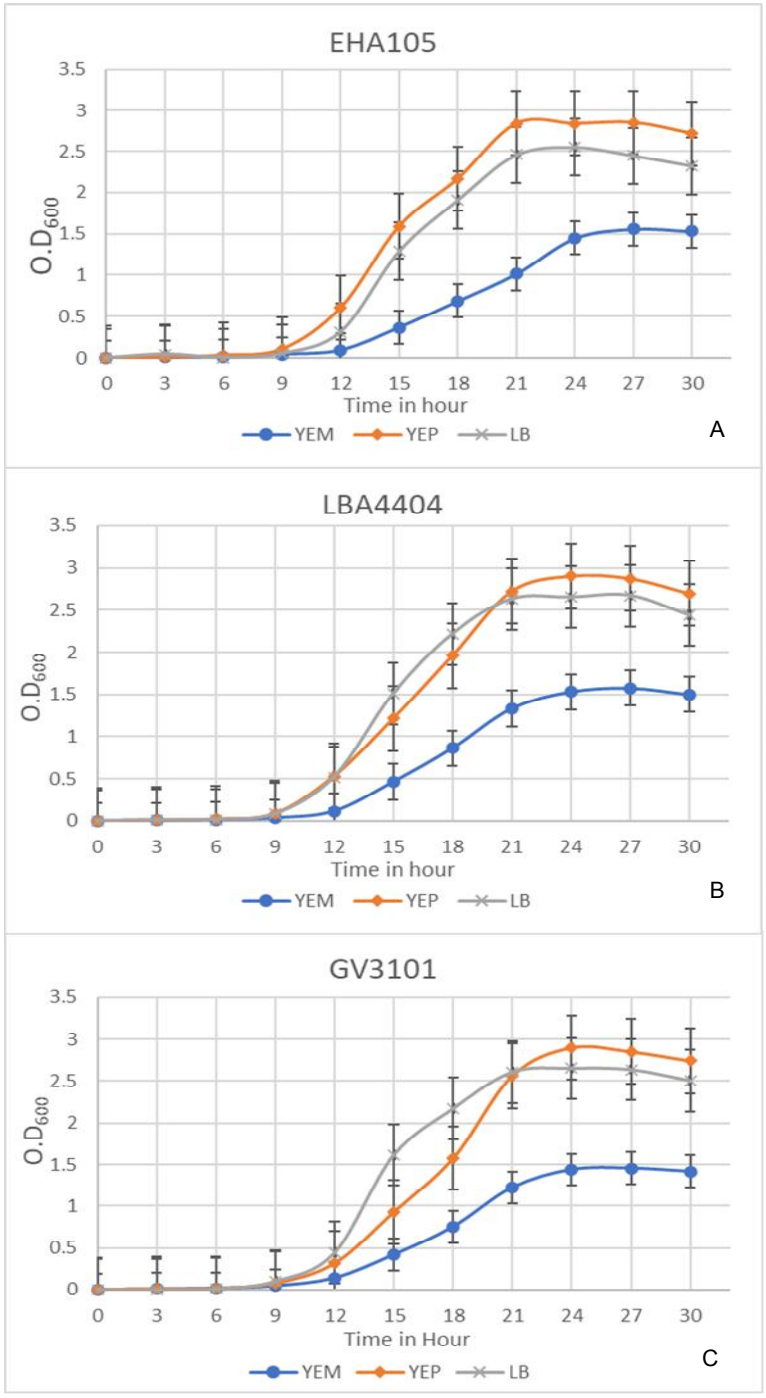

Fig. 1. Growth Curve for (A) EHA105, (B) LBA4404, and (C) GV3101 strain

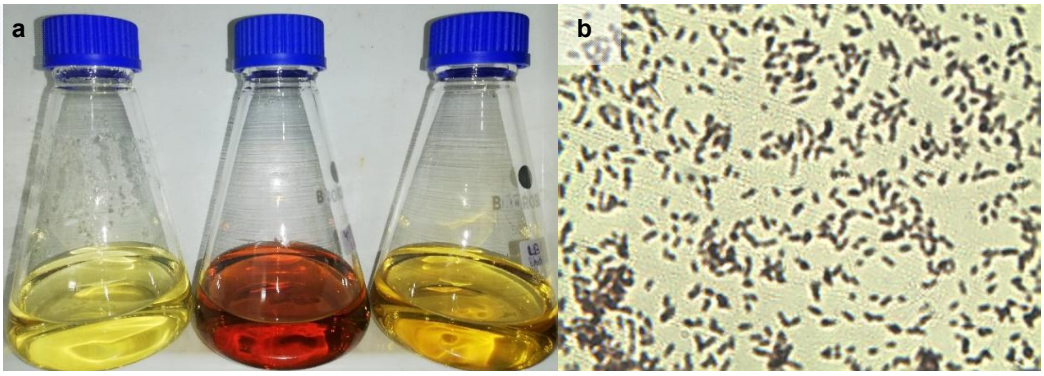

Fig. 2. (a) Nutrient broth (from left) YEM, YEP, LB (b) Morphology of rod-shaped bacteria after Gram staining under 40x objective microscope

Table 2. Growth data of three strains of *Agrobacterium* over three different nutrient broths at 24th hours

Nutrient media	O.D600 (Mean ± SE)		
	GV3101	EHA105	LBA4404
YEM	1.44 ± 0.017[c]	1.45 ± 0.010[c]	1.53 ± 0.006[c]
YEP	2.90 ± 0.047[a]	2.84 ± 0.007[a]	2.90 ± 0.055[a]
LB	2.65 ± 0.036[b]	2.55 ± 0.009[b]	2.65 ± 0.035[b]

Data represent mean ± SE, N=3, data is significant (P ≤ 0.01) Duncan's test.
Mean ± S.E. = Mean values ± Standard error of means of three experiments

5. CONCLUSIONS

This study was conducted to gain knowledge about the growth patterns of *Agrobacterium* in small batches of cultures media. As *Agrobacterium* is widely used for genetic transformation of plants and crop improvements, so knowledge of its growth patterns and optimal O.D. or cell suspension is necessary for the effective infection of plant material. It was seen that YEP media favors the growth of *Agrobacterium* more in comparison to LB and YEM media. This could be due to the different sources of nitrogen and mineral in all three mediums, YEP is supplemented with peptone whereas LB media has tryptone and YEM contains mannitol. This study can help the researchers to gain knowledge of the growth pattern of *Agrobacterium* to be used in the genetic transformation of plant species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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