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Optimization of NPK Rich Tree Leafy Substrate Blends for Enhanced Mushroom Yield using Response Surface Methodology

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Abstract: Mushroom production creates large amounts of a byproduct, mushroom substrate (MS), which has potential bio-environmental uses and agricultural purposes. The effectiveness of NPK rich tree leafy substrate blends for promoting mushroom apex growth was assessed using Response Surface Methodology (RSM) of which a Box-Behnken Design (BBD) was adopted. Of the five substrate blends tested, the NPK-rich leafy substrate and the 25%A: 75%B blend were significant in promoting development of apex ($p < 0.05$). The optimal quadratic model obtained was statistically significant ($p = 0.0136$) and the predictive power was high ($R^2 = 0.9002$). The maximum apex weight (1.765 g) was attained in Run 22 with even substrate ratios, and growth was suppressed in Run 38 due to excessive mixing. Synergetic effects were realized in the 50:50 blend, and contour plots showed that apex development worked best at intermediate nutrient level, suggesting a balanced substrate composition is most critical. Such findings, which emphasize the relationship between agro-waste recycling and increased mushroom yield (in terms of apex growth and vitamin D_2 content), are options for small-scale mushroom cultivators.

Index Terms - Mushroom substrate, Response Surface Methodology, Box-Behnken Design, Apex Growth, Substrate Optimization.

I. INTRODUCTION

From an environmental perspective, mushroom cultivation is considered an eco-friendly biotechnological process that consumes agro-industrial and agricultural by-products to provide high-quality edible and medicinal fungi. These mushrooms are characterized by their high-quality proteins, essential vitamins, polysaccharides, and numerous bioactive compounds with therapeutic activities (Chang & Miles, 2004). One of the most important determinants of mushroom yield and quality is the selection and formulation of the cultivation substrate. Conventionally, substrates are composed of the lignocellulosic sources like straw, sawdust, and husks; nevertheless, such resources frequently possess a high C:N ratio, which may restrict microbial activity and the availability of nutrients for basidiomycetes (Hoa et al., 2015). To replace the synthetic fertilizers with cheaper sources of nutrients and improve the performances of the substrates, the use of agricultural waste rich in NPKs is emerging as a potential solution (Philippoussis, 2009). Substrates like poultry manure, cow dung, green biomass and composted crop residues exhibit better nutrient balance, biological activity and moisture retention which are important parameters for sustenance of healthy mushroom growth and fruiting body (Rupesh et al., 2021). Through the use of these NPK rich substrates, growers could dramatically increase mushroom production, and in the process close the loop on farm waste. It is of great significance to design suitable formula of the NPK rich agricultural residues which can maximize the yield benefits from the resulting substrate (Royse et al., 2007), by understanding thoroughly the complicated interactions among the physical, chemical, and biological variables in the substrate. Classical experimental procedures, such as the One-Factor-At-a-Time (OFAT) approach, are common practices because they are easy to perform. However, single-variable

‘‘OFAT’’ experiments are relatively narrow in scope since they do not account for variable interactions, and therefore tend to produce non-efficient and non-reproducible results (Montgomery, 2017).

On the other hand, the contemporary statistical optimization methodologies like the BBD as a part of RSM, provide solid and powerful grounds to the premises which may be used for optimization of substrate formulations in mushroom cultivation (Anderson & Whitcomb, 2016). BBD is particularly useful when the number of variables is moderate and quadratic (second-order) relationships are the objective in terms of amounts and levels studied without the need to perform a full factorial experiment, thus decreasing the number of experimental runs but the significant interactions are taken into account (Ferreira et al., 2007). This methodology represents attractive choices in the enrichment of substrate supplements with NPK for an accurate screening of the nutrient ratio that favors the highest growth of the fungal biomass and fruiting body yield (Bezerra et al., 2008). More particularly, because design points are located at process space edge midpoints (not the edges themselves) in preference to process space extremities, BBD substantially reduces the likelihood of experimenting at naive or extreme conditions. It also helps to generate predictive models and response surface plots used in decision-making involved in substrate formulation and process control (Montgomery, 2017). The effectiveness of BBD in several agro-biotechnological applications including optimization of the substrate condition supports its application for the utilization of nutrient rich agro-wastes in mushroom cultivation (Bhak et al., 2005). Utilization of NPK fecundated agro-waste such as leafy biomass, poultry manure, and legume crop wastes has been attracting attention because of their ability to supply balanced nutrients for mushroom mycelial development and increased yields. Recently, several work have been carried out to investigate the influence of various NPK-rich substrate formulations on productivity of the mushroom, mentioning that well-adjusted nutrient ratios of the substrate mixture can considerably provide a better biological efficiency and morphological characters including cap diameter and stipe length (Ayodele & Okhuoya, 2007; Hoa et al., 2015). The study highlights the significance of applying statistical optimization techniques, such as BBD, to design nutrient-rich substrate composition for enhanced mushroom growth.

The current work incorporates such a mosaic of biological, agronomic, and statistical resources that enables to measure individual influence of N, P and K-rich agricultural substrates on apex development which is a driving force of biomass and productivity in mushroom farming. Apart from traditional composting and simple supplementation, the present investigation focused on the nutrient balanced substrates prepared from NPK rich tree leafy biomass and agro-residues. Based on BBD in the context of the RSM, the effect of the specific nutrient ratio on apex growth was explored. In summary with the earlier, the inclusion of naturally NPK-rich substrates may solve the drawbacks of high C:N traditional substrates, improve nutrient availability, biological efficiency, and promote sustainable, low-cost practices in line with circular agriculture for small and mid-scale growers.

II. Materials and Methods

2.1 Statistical optimization using response surface methodology (RSM)

A BBD with the five factors, paddy substrate (1-100 g), NPK leafy substrate (1-100 g), 25% A and 75% B mixture (25-75 g), 75% A and 25% B mixture (25-75 g), and 50% A and 50% B mixture (0-50 g) was utilized to optimize the interactive effects of the five independent variables on the apex growth of the tested food mushroom. Each factor was studied at three levels as indicated in Table 1.

Table 1. Independent variables and levels used in Box-Behnken design

Factor	Variable Description	Unit	Minimum (−1)	Mean (0)	Maximum (+1)
A	Paddy substrate	g	1	50.5	100
B	NPK leafy substrate	g	1	50.5	100
C	25% A & 75% B mixture	g	25	50	75
D	75% A & 25% B mixture	g	25	50	75
E	50% A & 50% B mixture	g	0	25	50

BBD is especially useful for the studying quadratic response surfaces and applications of second order polynomial equations with as few experimental runs needed as possible (Montgomery, 2017). The design is economical in terms of time as fewer experiments are needed than for a full factorial design (Bezerra et al., 2008). The dependent variable, apex growth (g), was measured for each trial.

The second order polynomial model obtained from the fitting of the data was:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} DE + \varepsilon \quad \text{(Equation 1)}$$

The model (Equation 1) presented here was obtained by multiple regression and checked by analysis of variance (ANOVA). The model fit was confirmed through the validity of the statistical analysis (R^2 and the F-value) at 5% significance level. To explain the relationship of the response with various levels of the independent factors, contour plots were plotted by Design Expert (version 13.0, Stat-Ease, Inc., 2010).

2.2 Statistical approach for apex growth on NPK rich tree leafy mushroom substrate by BBD and RSM

BBD was used to arrive at the optimum value of apex growth parameter with the variables paddy substrate, NPK leafy substrate, and A & 75% B at 25% in 3 coded levels (−1, 0, +1) presented in Table 1. The design consisted in 46 experimental runs (8 factorial points, 6 central points and 6 axial points). Each experiment was done twice and average apex growth was taken as the response variable (Y). The experimental data were analyzed by "Design-Expert 13.0" software (Stat-Ease, Inc.).

2.3 Cultivation of *Pleurotus* spp for the determination of apex growth and content of vitamin D_2: A green house experimental study

The strain of *Pleurotus* spp. (test mushroom species considered statistical optimization of apex growth using BBD mode) used in this study was acquired from the Regional Agricultural Research Station's Division of Microbiology, Mushroom Research Laboratory in Tirupati, India. The culture was maintained on potato dextrose agar (PDA) slants and subcultured regularly. The sorghum grains were treated with the fungus to be used as spawn. The grains were boiled in a water bath for 15 minutes, and then they were mixed with calcium sulphate (2% w/w) and calcium carbonate (4% w/w). Three hundred grams of grain were placed in polythene bags that were 40 microns thick and had a 1000g capacity. After autoclaving for 30 minutes at 15 psi (pounds per square inch), the bags were let to cool to room temperature. After the sterile grains were combined with the mother spawn grains, they were cultivated for 20 days at 28±2°C. The grains were completely covered by white mycelium to create mushroom beds. The technique described by Bano et al., (1963) was used to cultivate *Pleurotus* spp. with modifications based on the results obtained from statistical optimization of NPK tree leafy substrate using BBD model within the framework of RSM. The *Swietenia macrophylla* (SM), *Gliricidia sepium* (GS) and *Sesbania grandiflora* (SG) NPK rich tree leafy biomass were mixed with the well-dried paddy straw (on a dry weight basis) in different proportions (25%, 50%, 75%, and 100% v/v plant leaves residues). The mixture was then chopped into pieces that were 2-3 cm long and left to soak in water for overnight. The slightly moist casing substrate was sterilized for 30 minutes at 121°C after the excess water was drained, and then it was left to cool to room temperature. The casing substrate was inoculated with 30 grams of spawn per kg. Following the making of tiny holes in the spawning beds, they remained 15 days at 28±2°C and 70±5% relative humidity in a dark chamber. The cropping room, which kept the temperature at 24±2°C and the relative humidity at or above 90%, was where the fully enclosed beds were relocated. Watering was done twice daily in the cropping room; the day before the first harvest, watering was skipped over. The first crop of mushroom fruit bodies was harvested after a period of 25 days. The fruit bodies were harvested after 60 days cycle and apex growth (empties) (recorded as gm of dry weight in weighing balance) and Vitamin D_2 content was measured.

2.4 Estimation of vitamin D_2

The spectrophotometric analysis of the samples were made using the procedure described in method applied by Perera et al., (2003) in which 0.5 g of each mushroom sample powder was weighed into 250 ml round bottom flasks, added 4 ml of sodium ascorbate solution (17.5 g sodium ascorbate in 100 ml of 1 M NaOH), 50 ml ethanol and 10 ml of 50% potassium hydroxide. The mixture was refluxed and saponified for one hour at 80°C and was then cooled immediately to room temperature and poured into a separating funnel. The mixture was first extracted with 15 ml de-ionized water, then with 15 ml ethanol and finally with a serial n-pentane extraction consisting of 50, 50 and 20 ml, respectively. The combined organic layers were repeatedly extracted with three portions of 50 ml of 3% KOH in 5% ethanol and finally with deionized water until the washings were neutral. The organic layer was exchanged to 5 ml of ethanol and evaporated to dryness at 40°C and re-dissolved in 5 ml of ethanol. The sample was filtered through a 0.45 µm non-pyrogenic filter. In this study, the vitamin D_2 then determined by spectrophotometric methods of Saad (1978) method where calciferol that reacts with 11N hydrochloric acid in the presence of symmetrical tetrachloroethane to develop greenish yellow and maxim absorbance at 440-460nm. 2ml aliquots of the prepared samples were evaporated to dryness on a boiling water bath. Following this, 1 ml of 11N hydrochloric acid and 1ml of symmetrical tetrachloroethane were added and the tube warmed for 10minute on the water bath with shaking at intervals. The volume was made up to 7 ml with acetone and after cooling, the absorbance was measured with spectrophotometer by keeping a

reference blank solution (unirradiated sample solution during the growth) in the cuvette in the spectrophotometer. The absorbance of the reference blank was measured at 450 nm. The blank was subtracted and the cuvette with sample solution was placed in the spectrophotometer and the absorbance read at 450 nm.

III. Results and Discussion

3.1 Statistical optimization using response surface methodology (RSM)

In order to maximize the apex growth of the test mushroom species, RSM based BBD was adopted after conducting preliminary screen using OFAT (Montgomery, 2001). The OFAT method was successful in identifying the most important factors affecting apex growth. Five substrate compositions were found to be strong contributors to apex growth - paddy substrate, NPK leafy substrate, 25% A + 75% B mixture, 75% A + 25% B mixture and 50% A + 50% B mixture. These were used as independent variable in the BBD, with each factor tested at three levels, in order to optimize the level of the factors and to study the individual and combined effect of these on apex growth (Khuri and Cornell 1987). This statistical process successfully identified the most favorable combinations of substrates to maximize apex growth of the organism. Table 2 includes the BBD experiment plan and the actual and predicted apex growth responses based on laboratory experiments. The impact and interaction of the five factors on apex growth were clearly revealed through a second order polynomial regression equation model.

Table 2. Experimental design and results of BBD

Run	A: Paddy substrate (g)	B: NPK leafy substrate (g)	C: 25% A & 75% B (g)	D: 75% A & 25% B (g)	E: 50% A & 50% B (g)	Response: Apex growth (g) (Actual)	Response : Apex growth (g) (Predicted)
1	50.5	50.5	75	25	25	0.678	0.651
2	1	1	50	50	25	0.345	0.337
3	50.5	50.5	75	50	0	0.897	0.873
4	50.5	100	50	50	0	1.234	1.198
5	50.5	100	75	50	25	0.567	0.546
6	50.5	1	50	75	25	0.897	0.876
7	50.5	50.5	25	50	0	1.167	1.150
8	100	50.5	50	50	50	1.453	1.430
9	50.5	50.5	25	50	50	1.345	1.330
10	50.5	50.5	25	75	25	0.675	0.665
11	1	50.5	25	50	25	0.432	0.421
12	100	50.5	50	25	25	0.897	0.895
13	50.5	50.5	75	50	50	1.067	1.058
14	50.5	50.5	50	50	25	0.543	0.534
15	50.5	1	50	50	0	0.567	0.554
16	50.5	50.5	50	25	50	0.893	0.882
17	50.5	50.5	50	75	50	0.765	0.764
18	50.5	50.5	50	50	25	0.435	0.434
19	100	50.5	50	50	0	0.332	0.321
20	50.5	50.5	25	25	25	1.564	1.550
21	1	50.5	50	75	25	0.897	0.883
22	100	50.5	25	50	25	1.765	1.724
23	1	50.5	50	50	0	0.678	0.662
24	50.5	1	75	50	25	0.564	0.559
25	1	50.5	50	25	25	0.765	0.753
26	100	100	50	50	25	1.003	0.992
27	50.5	100	50	50	50	0.987	0.974
28	50.5	1	50	50	50	0.345	0.336
29	50.5	50.5	50	50	25	0.321	0.314
30	50.5	1	25	50	25	0.897	0.885
31	50.5	50.5	50	75	0	0.564	0.556
32	50.5	1	50	25	25	0.321	0.313

33	50.5	100	50	75	25	0.432	0.427
34	1	100	50	50	25	0.786	0.773
35	50.5	50.5	50	25	0	0.546	0.532
36	50.5	50.5	75	75	25	0.897	0.880
37	50.5	50.5	50	50	25	0.213	0.209
38	100	50.5	50	75	25	0.167	0.161
39	100	50.5	75	50	25	0.908	0.895
40	50.5	100	50	25	25	0.876	0.869
41	100	1	50	50	25	0.432	0.427
42	1	50.5	50	50	50	0.567	0.555
43	50.5	50.5	50	50	25	0.213	0.208
44	50.5	50.5	50	50	25	0.876	0.854
45	1	50.5	75	50	25	0.432	0.429
46	50.5	100	25	50	25	0.890	0.860

Out of 46 such trials, Run 22 exhibited maximum apex biomass (1.765 g) (Table 2), which was prepared with paddy substrate (100 g), NPK rich leafy substrate (50.5 g), and mixed substrate (25 g) with the following compositions under 25% A & 75% B, 75% A & 25% B, and 50% A & 50% B were found that such mixture provided suitable carbon to nitrogen ratio, porosity of the substrate, moisture availability which was the facilitating factor for efficient utilization by a kind of microorganisms which helped in colonizing the roots and the apex to spread. Run 38 in contrast that used levels of base paddy and NPK leafy substrates but increased levels of the mixed substrates (viz., 50 g of 25% A & 75% B and 75 g of 75% A & 25% B) had notably inferior growth in apex (0.167 g) suggesting the application of excess substrate leading to compaction, trapping moisture which may have suffocated the composting process resulting in slower microbial respiration and nutrient availability This demonstrates the importance of tight substrate ratio refinement, as relatively small compositional changes may have significant effects on biological performance. Based on the diagnostic plot for quadratic model of apex growth in mushroom cultivation approves the quality and correctness of the model formulation. The spread of studentized residual through fitted values display an almost random and symmetric pattern around zero and do not exhibit any obvious funnel pattern or curvature. The above indicates that the model satisfies the basic regression assumptions of homoscedasticity and linearity that are necessary for making valid statistical inferences (Montgomery, 2017). The majority of residuals fall within ±2 units, indicative of a good model fit between the observed and predicted data, as seen with the high R^2 (0.9002) and adjusted R^2 (0.9203) resulting from ANOVA (Figure 1) (Box et al., 2005).

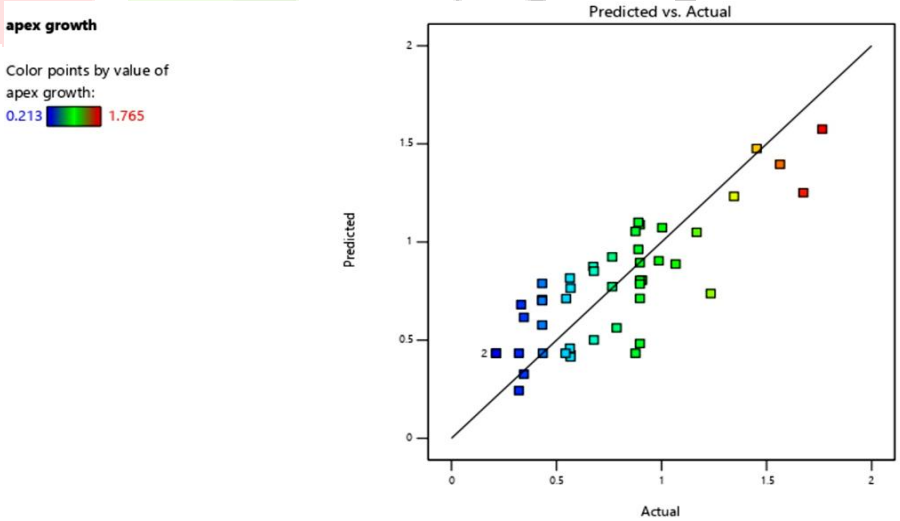

Figure 1: Actual and predictive values of apex growth

In addition, while the color gradient (representing Cook's distance or leverage values, presumably) shows a small number of points in slight moderation (tending towards red), this suggests that no one point is too excessive in any influence on the model. Values of Cook's distance less than 1 are usually regarded as non-influential (Belsley et al., 1980), which is indeed the case here with most of the observations. While the occurrence of a few high-leverage points is often understood in biological

experiments using a variety of substrate compositions, it does not in and of itself invalidate the model, especially in light of this overall distributional balance as evidenced by the diagnostics. More importantly, the fact that there are no systematic patterns in the residuals also speaks to the reliability of the regression model as a whole in interpreting main effect term such as factor B (NPK leafy substrate), factor C (25% paddy + 75% NPK) and the interactions of combinations thereof with other substrate mixes. This observation is consistent with previous observations where nutrient-rich nitrogenaceous substrate and/or optimal C:N ratios are important in stimulating robust fungal growth (Royse et al., 2004).

3.2 Analysis of variance (ANOVA) for the quadratic model: Validity and fit

The results of ANOVA generated a regression equation representing the response of the five independent variables. In the present study, a quadratic model was fitted to the data by least squares with all terms, including nonsignificant terms. We obtain the following equation:

Y (apex growth) = +0.4335 + 0.1285A + 0.1504B - 0.1703C - 0.0777D + 0.0896E + 0.0325AB - 0.2142AC - 0.2154AD + 0.3080AE + 0.0025BC - 0.2550BD - 0.0063BE + 0.2770CD - 0.0020CE - 0.0357DE + 0.1155A^2 + 0.0558B^2 + 0.3559C^2 + 0.1131D^2 + 0.2442E^2 (Equation 2)

The ANOVA results of the quadratic model provided an overall spectrum of the way all different substrate ingredients affect mushroom apex growth and elucidated the important effect of single factor and even their interactions (Table 3).

Table 3: ANOVA of BBD of Apex growth

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	4.00	20	0.2001	2.56	0.0136
A-Paddy substrate	0.2641	1	0.2641	3.38	0.0778
B-NPK leafy substrate	0.3621	1	0.3621	4.64	0.0411
C-25% A & 75%B	0.4641	1	0.4641	5.94	0.0222
D-75% A &25 % B	0.0965	1	0.0965	1.24	0.2768
E-50% A & 50% B	0.1285	1	0.1285	1.65	0.2112
AB	0.0042	1	0.0042	0.0541	0.8179
AC	0.1836	1	0.1836	2.35	0.1377
AD	0.1855	1	0.1855	2.38	0.1357
AE	0.3795	1	0.3795	4.86	0.0369
BC	0.0000	1	0.0000	0.0003	0.9859
BD	0.2601	1	0.2601	3.33	0.0599
BE	0.0002	1	0.0002	0.0020	0.9647
CD	0.3069	1	0.3069	3.93	0.0585
CE	0.0000	1	0.0000	0.0002	0.9887
DE	0.0051	1	0.0051	0.0655	0.8001
A^2	0.1164	1	0.1164	1.49	0.2335
B^2	0.0271	1	0.0271	0.3476	0.5608
C^2	1.11	1	1.11	14.16	0.0009
D^2	0.1117	1	0.1117	1.43	0.2428
E^2	0.5203	1	0.5203	6.67	0.0161
Residual	1.95	25	0.0781		
Lack of Fit	1.63	20	0.0817	1.29	0.4227
Pure Error	0.3177	5	0.0635		
Corrected Total	5.95	45			

Fit Statistics			
Standard Deviation	2.40	R^2	0.9002
Mean	26.53	Adjusted R^2	0.9203
C.V. %	8.32	Predicted R^2	0.8050
Press	213.1	Adequate Precision	39.0863

The model was statistically significant (F = 2.56, p = 0.0136), indicating that the selected factors collectively contributed significantly to the observed variations in apex growth. The non-significant lack

of fit (p = 0.4227) suggested the model fits the data well, that is, the variation not explained by the model is due to random error rather than model inadequacy. The high R^2 indicated by 0.9002 to 0.8050 (adjusted and predicted R^2), as well as the high value of adjusted R^2 = 0.9203, suggested that the model is not only able to accurately describe the data, but can also give reliable predictions. This is very useful for new substrate formulations, indicating high internal coherence as well as external relevance. Adequate Precision of 39.0863, well above 4 (required for a robust model), showed the robustness of the model in the experimental range and its strength in controlling the parameters for the optimization of apex growth. From linear terms only NPK leafy substrate (factor B) was significant (p = 0.0411) in echoing the importance of nutrient enriched organic compost favouring fungal proliferation. NPK fertilizer is well recognized for root-providing nutrient elements among which nitrogen plays a central role that is essential for protein synthesis and cell division in fungus (Pathmashini et al., 2008). This is in agreement with a preliminary report of Royse et.al., (2004) where nitrogen supplementation was shown to enhance the biomass production and morphogenesis of *Pleurotus* species. Factor C, 25% paddy straw, 75% NPK substrate, was also significantly influential (p = 0.0222), probably as a result of the most appropriate ratio of C/N. Paddy straw is mainly composed of cellulose and lignin (carbon source), where as that of NPK is readily available nutrients. Also, the effect of the interaction between carbon and nitrogen sources may benefit mycelial growth, enzymatic activity and apex formation, as in the study by Kimenju et al., 2009), where balanced growth substrate greatly enhanced mushroom growth development. Most notably, the interaction of factor A (paddy substrate) and E (50%A+50%B) proved to be statistically significant (p = 0.0369), meaning an enhancing effect with a 1:1 combination of the two substrates. These may be attributed to enhanced aeration and water holding capacity owing to fibrous nature of paddy straw and the nutritional potency of the NPK substrate. Interactive effects related to these were also cited by Marcelo et al., (2019), who reported enhanced yield and quality of oyster mushrooms from straw-based substrate amended with organic fertilizers. The significance of the quadratic term, especially C^2 (p = 0.0009) and E^2 (p = 0.0161), signifies the presence of curvature in the response surface, and the meaning of those curvature on the response, representing non-linear effects of these factors on apex growth. This implies that there will be an optimum level of these substrate compositions and departure in either direction (excess or deficiency) could reduce performance. Non-linearity in biological systems is expected, particularly in growth processes such as these which have many interacting inputs (Rathore et al., 2021). These results indicate the importance of an accurate optimization of substrate formulations in order to attain the highest potential biological response. While many such other interaction and quadratic terms were not significant in their own right, they were preserved in the model to maintain and control its hierarchy and inquiring the possibility for combined effects of them as well as weak effects. This is in line with good modeling practices in RSM (Montgomery, 2017). However, the current study validated that the BBD approach as a powerful statistical procedure for optimization of biological systems having multiple interactions. Properly balanced combinations of paddy straw and NPK as leafy substrates can, result in mounting of apex and a sustainable alternative for mushroom cultivation. These observations are particularly pertinent in a circular agriculture perspective where agroresidues repurposed for high-value biochemical production would significantly alleviate waste and contribute to sustainable environment (Pathmashini et al., 2008).

3.3 Influence of independent factors and their interactions

In this research, a BBD was used to investigate the effects of five substrate factors and their interactions on the apex growth of mushroom. The quadratic term was significant in the model (F = 2.56, p = 0.0136) suggesting it is a good explanatory factor in describing apex growth response among the substrate blends. Significant for apex growth were the linear terms, B (NPK leafy substrate) and C (50% A & 50% B mix), both with p = 0.0411 and p = 0.0222, respectively. Among the AE interaction (paddy substrate × 50% A & 50% B mix) was also significant (p = 0.0369), implying that the mixed and base substrates combination may affect the growth of mushrooms. Furthermore, quadratic terms C^2 and E^2 were extremely significant (p = 0.0009 and p = 0.0161) denoting substantial curvature and non-linear influence of these variables on the growth of apex. These findings are consistent with the work of Yadav et al., (2019) who mentioned the improved enzymatic and morphological development of mushrooms when formulations on the basis of a combination of lignocellulosic substrate were well balanced. Also, Oscar et al., (1999) documented that, an appropriate level of nitrogen and structure of the substrate are required to promote mycelium colonization and fruit bodies, thus supporting the significance of factors B and C obtained in this study.

The RSM plot depicted in Figure 2 represents the interactive effects of Factor A (paddy) and Factor E (a 50:50 combination of paddy and NPK treatments) on the apex development of mushrooms.

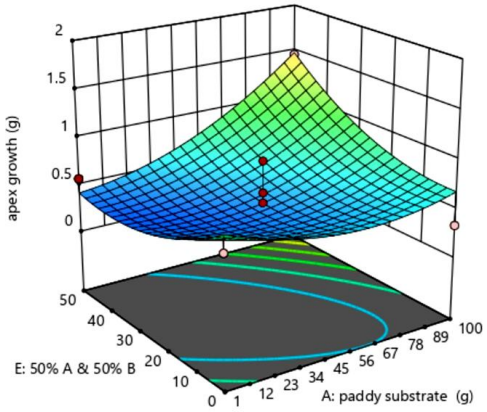

Figure 2: Interaction effect of AE

The curve of the graph is convex, which implies a synergistic effect of these two variables. Addition of paddy substrate with a higher level resulted in a significant increase in enhanced apex growth with very high levels of paddy substrate in 50: 50 mix (E). This indicated that the hybrid combination of the mechanical lodge resistance of the lignocellulosic paddy hull with the balanced nutrition profile of NPK rationed mixture appears to facilitate the ideal environmental setting for mushroom growth and apex elongation. Paddy straw has a high content of cellulose and hemicellulose, which will give rise to a strong network structure to support fungal growth, thus contributing to optimum aeration and water holding capacity (Royse et al., 2004). But it may be low in available nitrogen for best growth. On the other hand, the NPK leafy substrate which contains nitrogen, phosphorous and potassium act as strong nutrient boosters (Ayodele & Okhuoya, 2007). The change in the 50:50 mix effectively fulfills in this gap in nutritive value, helping make substrate quality better without sacrificing structure. Such a balance is essential since over nitrogen can also suppress fruiting process by promoting proliferated vegetative growth (Chang & Miles, 2004). Therefore, the curvature and apex growth of the 3D surface verify that the optimal nutritional stimulation through E supplemented with a high level of A, was the most favorable condition for mushroom apex enlargement. This interaction is also consistent with results of Royse et al., (2007) who indicated that substrates containing a combination of carbon rich and nitrogen rich materials result in higher mushroom productivity because of improved enzymatic degradation and increased rate of nutrient uptake. Furthermore, the same interaction pattern has been frequently observed in studies optimizing substrates for *Pleurotus* spp. and *Volvariella* spp., growth was enhanced by the use of mixed organic residues instead of single source substrate (Pathmashini et al., 2008; Hoa et al., 2015).

The RSM plot in Figure 3 depicts the relationship between Factor B (NPK leafy substrate) and Factor D (75% paddy (A) + 25% NPK leafy (B) formula) effecting the growth of mushroom apex.

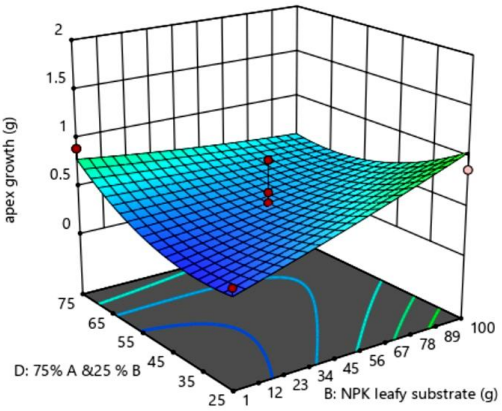

Figure 3: Interaction effect of BD

The graph showed an increment in apex growth when B and D are fairly-high to medium, indicating a synergistic effect that boosts the condition ideal for fruit body formation. This data is congruent with the notion that a mycorrhizal partner does not favor ample or deficient nutrient, but rather that moderate nutrient supply enhances healthy mycelial activity and apex growth. NPK leafy substrate also provides necessary macronutrients i.e., NPK which are fundamental for fungal metabolism, protein synthesis, and energy transformation processes (Royse et al., 2004; Hoa et al., 2015). Nonetheless, excessive NPK can result in substrate toxicity or imbalance, thus limiting mushroom productivity and quality (Chang &

Miles, 2004). Consequently, an intermediate level of B guarantees the sufficiency of nutrients without an excessive burden on fungal physiology. Meanwhile, Factor D which contained 75% paddy and 25% NPK, represents a structural substrate supplemented with the least possible nutrients to enhance increase in degradation of substrate and water retention capacity thereby enhancing penetration and apex growth of mycelium, and the best growth of mycelium (Pathmashini et al., 2008). The surface gradient pattern in the graph indicates that the response tends to form a dome shape, implying that there is an optimum level at which further increases in the particular factor would not necessarily provide substantial additional acceleration of growth, which can be explained by the law of diminishing returns in substrate enrichment. This is in agreement with results reported by Bich et al., (2021) & Salama et al., (2019) where balanced addition of balanced organic residues and nutrient supplements individually demonstrated marked increase in fungal biomass and fruiting reactions. It is also in agreement with previous findings of Pathmashini et al., (2008) who reported that the supplementation of sawdust with nitrogen supplements increased *Pleurotus* spp. yield and morphology. Therefore, the trend in Figure 3 supports the hypothesis that the balanced inclusion of lignocellulosic structure (paddy straw) and mild nutrient fortification (NPK) is beneficial for successful formation of the mushroom apex, leading to overall higher productivity while preserving substrate utility.

This RSM plot which shows the interaction of Factor C (substrate mixture of 25% A & 75% B) and Factor D (substrate mixture of 75% A & 25% B) with respect to mushroom apex growth was given in Figure 4.

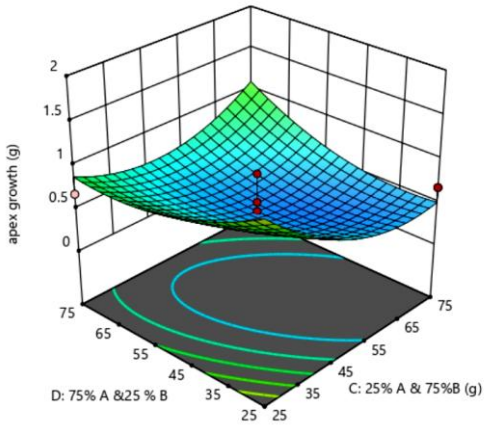

Figure 4: Interaction effect of CD

The plot also exposed a bimodal trend, where apical growth is increased at each of the extremes of the substrate combinations, suggesting that separate substrate balances can each optimize apical growth, albeit under different conditions. The 25% A (paddy) and 75% B (NPK higher leafy substrate) is a probable that would offer nutrient enriched medium for more enzymatic activity and a faster hyphal growth for high content of nitrogen and mineral (Hoa et al., 2015). Perhaps due to the high nutrient amounts, the stages of this schematic can be an advantage for both the initial growth and apex extension. However, a high level of B might also decrease porosity and increase compaction and water-holding capacity that may lead to decreased aeration and fruiting in some cases (Royse et al., 2004). Alternatively, the 75% A and 25% B mix would encourage aeration, structure and water holding stability which are also paramount for fruit bodies formation and apex integrity (Chang & Miles, 2004). Paddy straw is rich in lignocellulosic fibrous structure that prevents complete mycelial colonization, and provision of enough air circulation along with mechanical support for fruit body growth (Pathmashini et al., 2008). This blend helps keep the physical substrate environment ideal for mycelial health, even if the nutrients are a step-down from ideal. That the resulting extreme forms a saddle-shaped surface endorses the concept of conditional optimization: the CB strategy may be effective on a certain substrate during a first phase of the mushroom development while the CA strategy can be effective on the same substrate during a second phase of the mushroom growth cycle. This reveals a non-linear synergism between the composition of the substrate and the development of the apex, in line with the observations of Hoa et al., (2015) who found that while both a high nutrient and high structure substrate could act to allow mushroom development, they did so through different mechanisms. This suggests that both substrate type combinations (25 : 75 and 75 : 25) are capable of supporting the development of healthy apices but through different mechanisms one involving a biochemical (nutrient enrichment) and one involving a physical (substrate structure) stimulant. This underscores the need for substrate customizations according to the particular developmental objectives of mushroom cultivation.

3.4 Conformity of the test results using BBD model

A clear comparison was noted in the accuracy of the model and the biological response in Runs 22 and 38 of the RSM designed experiment. Run 22 with 100 g of paddy substrate, 50.5 g of NPK leafy substrate, and partial proportions of mixed substrates (25 g of 25%A:75%B, 50 g of 75%A:25%B, different proportions of 50%A:50%B, resulted in an observed apex growth of 1.765 g, and model prediction of 1.724 g though with the computed error percentage of 2.323%, exhibiting a good agreement in trend if not the same (Table 4).

Table 4: Methods validation test results

Run	A: Paddy substrate (g)	B: NPK leafy substrate (g)	C: 25% A & 75% B (g)	D: 75% A & 25% B (g)	E: 50% A & 50% B (g)	Response: Apex growth (g) (Actual)	Response : Apex growth (g) (Predicted)	Error (%)
22	100	50.5	25	50	25	1.765	1.724	2.323
38	100	50.5	50	75	25	0.167	0.161	3.593

These low error margins indicated that high predictive accuracy as well as the model's good fit with experimental data under optimal or near-optimal conditions (Myers et al., 2016; Montgomery, 2017). By contrast, apex growth was observed to be significantly lower (0.167 g) in Run 38 as compared to that in Run 36 (0.194 g) when the primary substrates were equivalent while higher isomeric dose was provided (50 g of 25%A:75%B and 75 g of 75%A:25%B); the model predicted it to be 0.161 g for Run 38 with a higher error (3.593%). This indicated the model's poor extrapolated prediction of the combinations involving very high proportions of mixed substrate which may be caused by unmodeled inhibitory mechanisms like excessive water holding, compaction or nutrient imbalance (Royse et al., 2004). But the significant prediction deviation of Run 38 indicates that lack-of-fit may be resulted under substrate extremes, a general phenomenon in biological systems where nonlinear responses and saturation effects can distort quadratic predictions (Montgomery, 2017). This stresses the importance of improving model robustness through consideration of the cubic or interaction terms or augmenting the experimental design near the boundaries (Myers et al., 2016). Nevertheless, or for higher precision, prospective research could include more heterogeneous points in the design space and higher-order RSM models.

3.5 Estimation of apex growth and vitamin D_2 in *Pleurotus* spp.

Vitamin D_2 content and apex growth in *Pleurotus* spp. in response to different substrate formulations was also determined in this research. The highest apex growth (1.72 g (DW) dry weight) and vitamins (Vitamin D_2: 17.5 µg/g DW) were achieved by Substrate C which is intelligent to combine both structural lignocellulosic material and NPK rich tree leafy biomass (Table 5). This optimal balance probably increases C:N ratio and micronutrient availability of the compost which are important for increased mushroom growth and fruit body formation (Chang & Miles, 2004).

Table 5: Influence of substrate compositions on apex growth and vitamin D_2 content

Substrate Code	Substrate Composition	Apex Growth (g in DW)	Vitamin D_2 Content (µg/g DW)
A	Paddy substrate 100% (g)	0.73	8.1
B	NPK leafy substrate 100% (g)	1.01	10.2
C	25% A & 75% B	1.72	17.5
D	75% A & 25% B (g)	1.45	15.3
E	50% A & 50% B (g)	1.28	12.7

The reasonable performance of Substrate C was in agreement with the prediction of Run 22 of Box-BBD, a well-known and efficient method to optimize several factors using few experimental runs (Aninda et al., 2024). The capability of BBD for the detection of non-linear effects in substrate sources was especially helpful to identify the best combination for both biological yield and nutrient yields maximization. On the contrary, substrate A (100 % paddy straw) showed also the lowest apex development (0.73 g DW) and the lowest Vitamin D_2 concentration (8.1 µg/g DW). This finding is probably due to high lignocelluloses nature and low nutrient content of straw-based substrates that can effect on enzymes degradation and release of nutrient for effective mushroom growth (Akcay et al., 2023). When growth (1.01 g DW) and vitamin content (10.2 µg/g DW) were compared in substrate B (100% NPK-rich leafy biomass), it was confirmed that a nutrient supply rate for fast growth is achieved

by sufficient nutrient availability, but the physical condition of the substrate also plays a vital role for optimum growth. The intermediate responses of the mixed compositions (D and E) further confirm the idea of a balanced combination of nutrient-dense leafy substances and fibrous straw conducive to apex growth. It is also notable that Vitamin D_2 levels are elevated in the absence of ultra violet (UV) exposure. Although ergosterol is a precursor of Vitamin D_2 and usually requires exposure to UVB for its conversion, however due to the effect of substrates rich in magnesium and zinc available in leafy biomass used in the expression of ergosterol, it could potentially stimulate ergosterol biosynthesis and result in an increased basal presence of Vitamin D_2 (Phillips et al., 2011; Mattila et al., 2002). The utility of BBD was demonstrated to facilitate and develop effective detection of such nonlinear interactions, and the empirical relationships using reduced experimental efforts. Finally the present work is to not only prove the efficacy of using RSM for optimizing substrate formulation but also to show and develop a reasonable and attractive valorization route for NPK rich tree leafy biomass in an eco-friendly manner, which can have substantial importance for waste utilization industry and in bioprocess engineering.

IV. Conclusion

The BBD was used for RSM to optimize substrate formulations for improving the apex growth of mushroom. Major findings were the NPK enriched leafy substrate and 25:75 paddy to NPK blend enhanced apex development. The statistical model established was solid (R^2 of 0.90) including separate and interaction effects with minimal lack-of-fit. Findings highlighted the need of a balanced composition of the substrate, including nutrient content and aeration. The study supported eco-friendly sustainable substrate design with the waste of agricultural residues such as the NPK rich tree leaf biomass for potential scale up practices for mushroom production and optimization of bioprocesses. For further studies, it is recommended to address NPK enriched substrates in long-term yield trial and scalability. Focus on environmental assessments and regional adaptation would provide better applicability in circular mushroom farming systems.

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