American Journal of Applied Mathematics and Statistics, 2018, Vol. 6, No. 6, 232-238
Available online at http://pubs.sciepub.com/ajams/6/6/3

DOI:10.12691/ajams-6-6-3

Selection of Potent Isolates from a Population of *Alternaria Alternata*, a Leaf Spot Pathogen of Poplar

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Received August 17, 2018; Revised October 01, 2018; Accepted November 09, 2018

Abstract Poplar, an important tree in the agri-silvicultural system, is propagated mainly through cuttings to maintain genetic purity. Monocultures of poplar clones are amenable to many diseases as they have a narrow genetic base. Pathogenic populations have variability in terms of pathogenicity and virulence which are governed by its genetic makeup. Mapping the variability and selection of potential pathogenic isolates for breeding disease resistance remains a challenge. During the survey conducted in poplar nurseries located at different geographical sites, altogether 72 isolates of *Alternaria alternata*, were collected from four commercial clones of *P. deltoides*. Three selection methods were attempted to select fifteen potent *A. alternata* isolates based on growth rate, sporulation and spore size (maximum length and maximum breadth). Initially, Rough Gauging Method which is simply based upon index of sum of the character's values and Equal Class Interval Method which depends upon the index of class interval scores were applied. To overcome the limitations of the above two methods, Unequal Class Interval Method was proposed based on Coefficient of Variation for each character assessed. The index was constructed using the geometric rather than arithmetic mean as the former normalizes the range, so that, no range dominates the scores assigned to the characters. The proposed method is recommended for the situations when the criterion variable depends upon various growth characters having inherent significant variation among each other.

Keywords: *agri-silviculture, class interval, clones, coefficient of variation, geometric mean, order statistic*

Cite This Article: Kartik Uniyal, Girish Chandra, R.U. Khan, and Y.P. Singh, "Selection of Potent Pathogenic Isolates from a Population of *Alternaria Alternata*, a Leaf Spot Pathogen of Poplar." *American Journal of Applied Mathematics and Statistics*, vol. 6, no. 6 (2018): 232-238. doi: 10.12691/ajams-6-6-3.

1. Introduction

Poplar, the most domesticated forest tree in India, has better synergy with agriculture system than forestry operations. It is propagated vegetatively through cuttings in order to maintain genetic purity. *Populus deltoides,* an exotic, have shown great promise in north-western part of the country. Use of single genotype (clone) over a large area entails enormous risk. Monocultures are widely believed to attract diseases and pests and to be more vulnerable than mixed stands, especially in long terms. Disease problems have, therefore, posed the question regarding the overuse of single clone and use of large monoclonal plantations [1].

The genetic structure of individual pathogen populations can vary through time and space as these populations evolve, or adapt, in response to local environmental changes [2]. The evolution of pathogens can be influenced by the type of resistance and the amount of diversity found in host populations and the type of cropping system. The year round availability of the host in field (for example, poplar) encourages the survival and persistence of the pathogen with high parasitic fitness.

For pathogens, the ability to infect hosts is the prerequisite of their survival and reproduction, and hence, high virulence should always be preferred. Yet, pathogen populations typically contain strains that are inferior in their pathogenicity traits compared to other co-occurring strains [3]. Genetic variation in patterns of host susceptibility and pathogen virulence and aggressiveness are essential underlying factors influencing disease epidemiology [4,5,6,7] and the emergence and spread of new diseases [8,9,10]. Therefore, the variation in pathogenic population is a pre-requisite to understand gene for gene co-evolution of the host and pathogen. In case of pathogen, the variation in its population must be understood on common denominators of vegetative and reproductive parts of its life cycle. Keeping this in background, the variation in the population of a potent pathogen of poplar, *Alternaria alternata* was studied based on growth, sporulation and spore size (length maximum (L_M) and breadth maximum (B_M)) of its isolates that were collected from four commercial clones, viz., G48, Udai, WSL22 and WSL39

from three locations spreading over a sufficiently large geographical area. However, mapping the variability of the pathogenic population and selection of potential pathogenic isolate remains a challenge. This challenge was attempted and resolved through a variety of statistical methods as described in following paragraphs.

2. Material and Methods

2.1. Survey of Poplar Nurseries

The surveys for collection of fungal isolates were conducted at four nurseries, viz., Bagwala, Udham Singh Nagar (29°30'N and 79°28'E), Maheshwari and Paniyala, Roorkee, (29°52'N and 77°53'E) and Thana Chappar, Yamuna Nagar (30°7'N and 77°18'E) of Wimco Seedlings Private Limited, India. The collections were made during the period 2007 to 2011. The different commercial clones of *P. deltoides,* namely, G-48, Udai, WSL-22 and WSL-39 were screened for *A. alternata* infection on leaves.

2.2. Collection and Isolation of Fungal Isolates

Leaves infected with *Alternaria* like symptoms were collected from each clone and put in paper bags and marked for the date, place and plant source (Figure 1). Thus, in all, 72 isolates of *A. alternata* were collected from four clones from different locations and years. The fungal character values are detailed in Table 1.

Table 1. Values of the growth characters of *A. Alternata* isolates

Isolate no.	Growth (cm)	Sporulation (no.x10^4/ml)	L_M (µm)	B_M (µm)	Isolate no.	Growth (cm)	Sporulation (no.x10^4/ml)	L_M (µm)	B_M (µm)
A1	5.92	643.33	32.83	15.43	A37	6.02	598.33	25.30	10.47
A2	5.85	520.00	36.83	14.63	A38	5.15	878.33	29.30	12.50
A3	5.97	693.33	31.57	15.90	A39	4.43	1101.67	27.13	12.80
A4	6.02	761.67	34.40	12.00	A40	7.00	981.67	28.20	13.57
A5	5.97	1020.00	27.93	13.23	A41	7.00	796.67	27.90	14.03
A6	5.50	741.67	30.87	15.27	A42	5.65	500.00	32.47	19.80
A7	6.52	1051.67	34.93	15.10	A43	6.50	645.00	26.67	13.70
A8	4.92	746.67	32.07	15.40	A44	4.48	765.00	29.20	13.30
A9	5.85	1075.00	25.73	13.03	A45	6.97	648.33	28.97	12.17
A10	5.73	1033.33	31.40	17.20	A46	5.45	520.00	33.43	12.00
A11	5.03	1180.00	29.50	14.67	A47	7.00	983.33	36.27	16.60
A12	6.73	1181.67	36.20	14.37	A48	6.05	331.67	29.70	13.27
A13	6.75	1173.33	31.57	15.03	A49	6.15	498.33	33.47	14.13
A14	5.63	880.00	37.40	13.77	A50	5.98	553.33	31.00	13.03
A15	6.57	1026.67	40.30	15.63	A51	6.45	646.67	34.77	12.77
A16	6.60	1161.67	28.07	14.97	A52	6.65	653.33	37.60	12.33
A17	6.07	776.67	22.20	13.73	A53	4.02	226.67	44.30	15.77
A18	6.10	695.00	33.40	13.17	A54	5.02	148.33	36.67	16.90
A19	6.77	941.67	29.60	12.13	A55	6.55	418.33	26.57	13.50
A20	6.45	741.67	22.03	11.63	A56	4.47	133.33	41.23	18.20
A21	6.43	646.67	32.07	17.23	A57	4.48	175.00	36.10	17.43
A22	5.93	531.67	32.17	10.97	A58	3.82	578.33	28.10	14.00
A23	5.88	638.33	30.70	14.43	A59	5.08	706.67	36.50	12.87
A24	6.67	916.67	33.17	11.83	A60	5.53	410.00	34.40	11.77
A25	7.00	956.67	30.17	13.10	A61	5.05	938.33	29.43	12.37
A26	6.62	763.33	36.50	14.60	A62	5.05	870.00	35.10	13.97
A27	5.45	600.00	28.57	12.70	A63	5.58	903.33	48.80	14.30
A28	6.32	496.67	31.40	11.87	A64	6.98	1043.33	35.83	16.77
A29	6.57	575.00	31.07	11.97	A65	7.00	1108.33	38.73	13.27
A30	6.07	441.67	24.60	12.03	A66	4.80	618.33	41.57	16.03
A31	4.15	490.00	40.60	13.93	A67	5.02	871.67	53.77	14.97
A32	7.00	895.00	32.00	13.17	A68	5.07	865.00	36.73	13.00
A33	4.47	588.33	33.00	14.33	A69	4.75	670.00	38.23	13.97
A34	6.28	351.67	31.50	14.37	A70	6.43	975.00	26.03	12.23
A35	6.35	615.00	35.53	12.03	A71	6.48	926.67	45.57	14.73
A36	6.38	551.67	30.03	15.07	A72	6.57	823.33	28.27	13.37

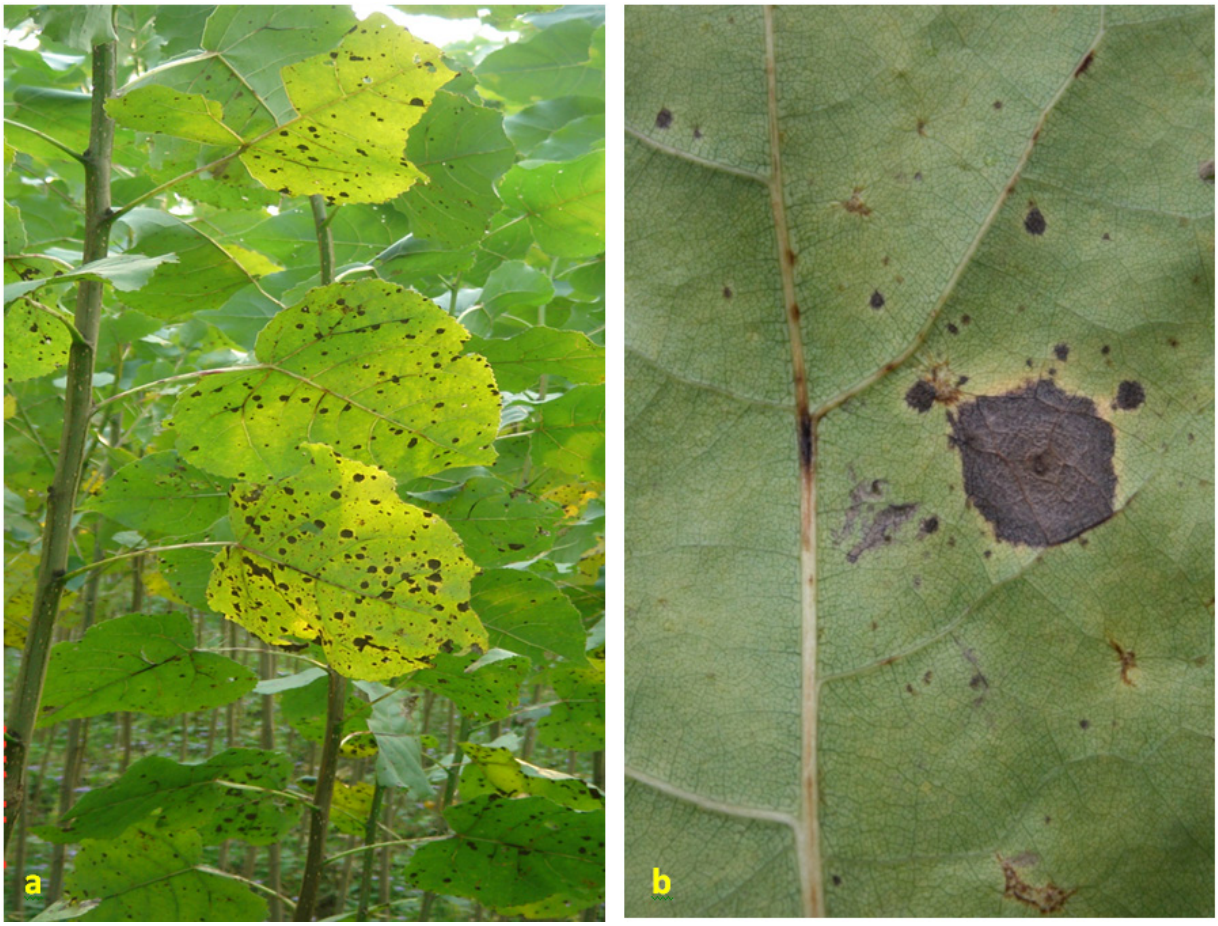

Figure 1. (a) Symptoms of *A. alternata* on poplar in nursery and (b) A close-up view of leaf affected with Alternaria leaf spot

2.3. Selection Methods for Isolates

Suppose N number (72 in the present case) of isolates of *A. alternata* were collected during the survey from the study area. These isolates depend on p growth characters (four, viz., growth rate, sporulation and spore size (L_M and B_M)) which were measured on the ratio scale. Based on isolate characteristics, the value of p recorded varied and random. The objective of the study was to select n number of isolates (15) out of the population having higher growth values. The selection criteria must be based upon appropriated index which reflects all the measured characters. Two methods for selection n from N isolates based on p growth characters [11] were initially used. The brief description of the steps was as follows:

2.3.1. Rough Gauging Method

Step I: Find an index I_1 for each isolate by adding corresponding values of p characters.

Step II: Arrange the I_1 values in descending order of magnitude.

Step III. Select n isolates having top order statistic.

2.3.2. Equal Class Interval Method

This method [11] was an attempt to overcome the limitations of the Rough Gauging Method.

Step I. Classify each character into equal number of class intervals (CIs) based on their corresponding range value. Each class interval had uniform width.

Step II. Assign score to each CI, for example, score 1 to first CI, score 2 to second CI, and so on.

Step III. Formulate the index (I_2) for each isolate by summing its corresponding scores.

Step IV. Arrange these indices into descending order of the magnitude.

Step V. Select n isolates having top order statistic.

2.3.3. Proposed Unequal Class Interval Method

In the present study, it was observed that each growth character had significant variation in their values due to genetic behavior. For example, the isolate number A7 and A66 when compared, usual % variation formula $\frac{(Max\text{-Min})}{Max}\times 100$, varied values for growth characters were observed (growth rate: 26.3%; sporulation: 41.2%; L_M : 16.0% and B_M : 5.8%). In another case, variations were extrapolated based on the range values of growth characters. For example, growth rate was observed 45.5%, sporulation 88.7%, L_M 59.0% and B_M 47.1%. Therefore, the score of an isolate, as per the above two methods, was not reflecting its true relative value. To make the variation homogenous, each character should be brought to a comparable uniform scale. Following steps were taken into consideration:

Step I: Arrange each character's value into either ascending or descending order of magnitude.

Step II: Classify each character's range into appropriate equiwidth CIs (n_S) based upon the Sturges [12] formula

$$n_S = 1 + 3.322 \log_{10} N \quad (1)$$

The number of classes for each character was determined, firstly, by calculating CV for each character. Suppose the calculated CVs were $V_1, V_2, ...V_p$. The average CV of all the characters may be taken as $\bar{V} = \frac{1}{p}\sum_{i=1}^{p} V_i$. Without any loss of generality, it was assumed that the appropriate number of CIs was n_S when CV is $\bar{V}$. Now, proportional allocation procedure was used for obtaining numbers of CIs for each character as:

$$\frac{C_1}{V_1} = \frac{C_2}{V_2} ... = \frac{C_p}{V_p} = \frac{n_s}{\bar{V}}. \quad (2)$$

Here, $C_1, C_2, ..., C_p$ are the numbers of CIs to be calculated for p characters.

Step III: Once the above C_i 's, $i = 1, 2, ...p$ were worked out, compound index was calculated assuming the matrix of growth character's value of N isolates as:

$$G = \begin{bmatrix} a_{11} \; a_{12} .....a_{1j} .....a_{1p} \\ a_{21} \; a_{22} .....a_{2j} .....a_{2p} \\ ..... \\ a_{i1} \; a_{i2} .....a_{ij} .....a_{ip} \\ ..... \\ a_{N1} \; a_{N2} .....a_{Nj} .....a_{Np} \end{bmatrix}_{N \times p} \quad (3)$$

Where, a_{ij} denoted the value of the j^{th} character of the i^{th} isolate. Supposing that R_j denoted the range of j^{th} character, therefore, the corresponding class width was $w_j = \frac{R_j}{C_j}$. The resultant classes for the j^{th} character would be:

$$b_{1j} = \left[a_{(1j)}, a_{(1j)} + w_j \right),$$

$$b_{2j} = \left[a_{(1j)} + w_j, a_{(1j)} + 2w_j \right), \ldots,$$

$$b_{C_j j} = \left[a_{(1j)} + \left(C_j - 1 \right) w_j, a_{(1j)} + C_j w_j \right)$$

where, b_{1j} denotes the 1st CI of j^{th} character and so on, $a_{(1j)}, a_{(2j)}, ..., a_{(Nj)}$ were the order statistics of the j^{th} character (means $a_{(1j)} = \min\left(a_{1j}, a_{2j}, ..., a_{Nj}\right)$ etc.) and [ ) represented the semi closed interval. These all the classes could be shown in the matrix form as:

$$G_1 = \begin{bmatrix} \begin{bmatrix} b_{11} \; b_{12} .....b_{1j} .....b_{1p} \\ b_{21} \; b_{22} .....b_{2j} .....b_{2p} \\ ..... \\ b_{i1} \; b_{i2} .....b_{ij} .....b_{ip} \\ ..... \\ b_{C_1 1} \; b_{C_2 2} .....b_{C_j j} .....b_{C_p p} \end{bmatrix} \end{bmatrix} \quad (4)$$

Step IV: The scores were assigned systematically to each CI in such a way that the score i designated to the class b_{ij} , $i = 1, 2, ..., C_j$, $j = 1, 2, ..., p$. The elements of matrix G could be converted into class interval scores (CISs) using G_1 as

$$\begin{aligned} a_{ij} &= 1, if \; a_{ij} \in b_{1j} \\ &= 2, if \; a_{ij} \in b_{2j} \\ &..... \\ &= C_j, if \; a_{ij} \in b_{C_j j} \end{aligned}$$

for any isolate i .

Step V: Finally, the compound index for each isolate was calculated by geometric mean of the CISs of each character of the isolate.

Step VI. Select n isolates having highest compound indices as calculated in step V.

3. Results

3.1. Survey of Poplar Nurseries

The year wise collection of the 72 *A. alternata* isolates was- 2007: 10, 2008: 8, 2009: 17, 2010: 35 and 2011: 2. Majority of isolates were collected from Uttarakhand (62) while only 10 isolates from Haryana state. Maximum number of isolates was collected from clone G48 (27) and minimum from Udai (7). Isolates collected from WSL series were practically same in number, i.e., WSL22 had 20 and WSL39 contributed 18 isolates.

3.2. Selection of *A. alternata* Isolates

3.2.1. Rough Gauzing Method

Range of growth of selected 15 isolates (A5, A7, A9, A10, A11, A12, A13, A15, A16, A39, A40, A47, A64, A65 and A70) was registered from 4.43 to 7.00cm. The sporulation of these isolates ranged from 975.00 to 1,181.70 x10^4 spores/ml. Likewise, the range for L_M and B_M were 25.73 to 40.30 µm and 12.23 to 17.20 µm, respectively.

3.2.2. Equal Class Interval Method

Four equi-width CIs were constructed for each character and assigned scores as per procedure (Table 2). The cumulative indices of selected 15 *A. alternata* isolates based on the sum of values of four fungal characters were ranged from 11 (isolate A16, A21, A25, A26 and A32) to 13 (isolate A15 and A64). Rest of the selected isolates had cumulative indices of 12 (Table 3).

3.2.3. Unequal Class Interval Method

The descriptive statistics of the population consisting of 72 isolates with four characters is shown in Table 4. In the proposed method, there were significant variations in the values of CV and, accordingly, the CIs also changed, for example, maximum CV (35.1%) was recorded for sporulation that had maximum number of CIs (12) also. In other characters, the CV remained quite close to each

other reflecting practically similar CIs. The sensitivity of the proposed method is reflected in CIs where it varies from 5 to 12 while in case of Sturges formula it remains constant to 7.

Table 2. Score assigned to the growth characters

Character	Class interval (CI)	Score assigned
Growth (cm)	3.0-4.0	1
	4.1-5.0	2
	5.1-6.0	3
	6.1-7.0	4
Sporulation (no.x10^4/ml)	100-400	1
	401-700	2
	701-1000	3
	1001-1300	4
L_M (µm)	20-30	1
	31-40	2
	41-50	3
	51-60	4
B_M (µm)	10-13	1
	13.1-16.0	2
	16.1-19.0	3
	19.1-22.0	4

Table 3. Cumulative index for the selected isolates of *A. alternata*

Isolate no.	Clone	Growth (cm)	Sporulation (no.x10^4/ml)	L_M (µm)	B_M (µm)	Cumulative index (I_2)
A7	WSL39	4	4	2	2	12
A10	Udai	3	4	2	3	12
A12	G48	4	4	2	2	12
A13	G48	4	4	2	2	12
A15	G48	4	4	3	2	13
A 16	WSL22	4	4	1	2	11
A21	G48	4	2	2	3	11
A25	WSL22	4	3	2	2	11
A 26	WSL22	4	3	2	2	11
A32	WSL39	4	3	2	2	11
A47	G48	4	3	2	3	12
A64	WSL39	4	4	2	3	13
A65	Udai	4	4	2	2	12
A67	WSL39	3	3	4	2	12
A71	Udai	4	3	3	2	12

Table 4. Descriptive statistics of a population of *A. alternata* isolates

Character	**CV (%)**	**Minimum value**	**Maximum value**	**No. of CIs (Sturges formula)**	**No. of CIs (Proposed method)**	**Class width**
Growth (cm)	14.3	3.8	7.0	7	5	0.64
Sporulation (no.x10^4/ml)	35.1	133.3	1181.7	7	12	87.36
L_M (µm)	17.4	22.0	53.8	7	6	5.29
B_M (µm)	12.8	10.5	19.8	7	5	1.87

Table 5. Description of CIs using unequal class interval method

Class no.	**Growth (cm)**	**Sporulation (no.x10^4/ml)**	**L_M (µm)**	**B_M (µm)**
1	3.8-4.5	133.3-220.7	22.0-27.3	10.5-12.3
2	4.5-5.1	220.7-308.1	27.3-32.6	12.3-14.2
3	5.1-5.7	308.1-395.4	32.6-37.9	14.2-16.1
4	5.7-6.4	395.4-482.8	37.9-43.2	16.1-17.9
5	6.4-7.0	482.8-570.1	43.2-48.5	17.9-19.8
6		570.1-657.5	48.5-53.8	
7		657.5-744.9		
8		744.9-832.2		
9		832.2-919.6		
10		919.6-1006.9		
11		1006.9-1094.3		
12		1094.3-1181.7		

As described above, the CIs were reflective of CV of a particular character. In case of sporulation, the range of values was relatively high (133.3 to 1181.7 x10^4/ml) and accordingly the CV (35.1%) that finally split it into equally high number of CIs of 12 when compared with other characters of the pathogen (Table 5).

3.3. Comparison of Selection Methods

Three methods were compared, and it was observed that nine isolates were common, viz., A7, A10, A12, A13, A15, A16, A47, A64 and A65 (Table 6). Four isolates (A12, A26, A67 and A71) were selected only in the Equal and Unequal Class Interval methods. In total, 13 isolates were commonly selected by the Equal Class Interval and Unequal class interval methods which indicated their proximity.

Table 6. Comparison of methods in terms of isolate selection

Rough Gauging Method	Equal Class Interval Method	Unequal Class Interval Method
		A 1
A5	-	-
A7	A7	A7
A9	-	-
A10	A10	A10
A11		
A12	A12	A12
A13	A13	A13
A15	A15	A15
A16	A16	A16
	A21	A21
-	A25	-
	A26	A26
	A32	
A39		
A40		
A47	A47	A47
	-	-
	-	-
-	-	A63
A64	A64	A64
A65	A65	A65
-	A67	A67
-		-
A70		-
-	A71	A71

4. Discussion

A total of 72 *A. alternata* isolates were collected; out of which, isolates from G48 were maximum in number (27 isolates) while, least number of isolates were from Udai (7 isolates). This may be due to the fact that G48 was most preferred clone and, accordingly, occupies largest share in the nurseries. Of 90 percent of the total planted poplar in north-western states of India, G-48 clone has highest share of about 36.2 percent among different poplar clones grown [13]. Moreover, it is highly susceptible to diseases. Also, in the present case, collection of isolates was limited with reference to locations as well as area covered within a nursery. This limitation may be ratified in future by multi-location surveys covering the entire region of poplar culture and proportionate coverage of planted area under each clone in a nursery keeping the thumb rule of 10 percent in mind. Then only, the collection of isolate be considered representative and exhaustive and may reflect the real population structure and variation of a pathogen. It may be useful in understanding host-pathogen interactions *per se* as well as testing new clonal material for resistance.

Selection of the most potent isolates out of a population was attempted. Fifteen *A. alternata* isolates were selected based on growth rate, sporulation and spore size (maximum length and maximum breadth), keeping the inoculum potential of a pathogen in view [14]. Initially, Rough Gauging and Equal Class Interval methods were applied for the isolate selection. On comparison, it was observed that only nine isolates were common. Despite overlapping of isolates in these methods, the index constructed by Rough Gauging Method is not reliable as negative correlations among different characters (four: growth vs. L_M; growth vs. B_M; sporulation vs. L_M and sproulation vs. B_M) dominated over positive ones (Two: growth vs. sporulation and L_M vs. B_M). Moreover, index is simply the sum of the values of the characters resulting to insensitivity to the order statistics of each of the character leading to improper selection of isolates. On the other hand, the Equal Class Interval method is more objective as value of each character recorded by an isolate has been given a relative score. Then, based on sum of these scores, the isolates were selected without any bias. However, no statistical rationality for the construction of number of classes for each character was followed in Equal Class Interval method. It led to improper scoring and, in turn, selection.

To further improve selection process of isolates, the Sturges formula was used that also constructed equal classes numbering seven, depending upon the population size (N) only, of each of the character studied. It could be helpful if isolates are selected based on only one character. If the CVs of the characters differ significantly from each other, Sturges formula is not appropriate for selection of isolates as score assigned to classes of the characters did not reflect the comparable real values. Therefore, the requirement of comparable uniform scale of scores of each character was felt. In the proposed method of Unequal Class Interval, the number of classes for individual character was based upon CV. The inclusion of CV is more helpful when there are different units of measurement of characters. The proposed method is like that of the Neyman's optimum allocation method used in stratified random sampling in which the sample size is directly proportional to the standard deviation of the respective stratum. In addition, preference to use geometric over arithmetic mean for calculating the cumulative index was due to the fact that the geometric mean normalizes the range being averaged, so that, no range dominates the scores assigned to the characters. If the cumulative indices were compared based upon the arithmetic and geometric mean, the values of score are not in the same order of magnitude. It means that there would be different isolates selected if arithmetic mean is applied.

Therefore, it can be concluded that the proposed method is helpful for isolate selection for the situations when the criterion variable depends upon various growth characters having inherent significant variation among each other (negatively correlation or poor positive correlation), as seen in the present study.

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